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Construction of an immunosensor for human cytomegalovirus infection diagnosis

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To my grandma

To my parents

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Resumo Alargado

O citomegalovírus humano (HCMV) é o maior vírus da família *Herpesviridae* e da subfamília *β-herpesviridae*. Como em todos os vírus herpes, a infeção pelo HCMV resulta no estabelecimento de uma infeção latente ao longo da vida do hospedeiro. Assim, sempre que uma pessoa é infetada, o vírus persiste num estado de latência celular, no qual as células infetadas não produzem nenhuma partícula infeciosa do vírus, mas retêm o seu genoma completo, tendo potencial para começar a produzir partículas virais mais tarde. Após infeção primária, o HCMV é excretado em fluidos corporais, como urina, sangue, saliva, lágrimas, secreções vaginais e cervicais, sêmen e leite materno. Este processo pode durar de meses a anos. Dessa forma, o HCMV pode ser transmitido por via oral, congénita, sexual, através da exposição à urina, por transfusão de sangue e transplante de órgãos ou medula óssea, sendo extremamente difícil identificar a sua via de transmissão.

O HCMV é considerado um vírus de paradoxos, pois este pode ser um potencial assassino ou um companheiro silencioso para toda a vida. Isto deve-se ao facto de a infeção pelo HCMV não induzir doença evidente em portadores saudáveis, devido a um controle imunológico efetivo, contudo a infeção pode ser grave e até fatal em indivíduos imunocomprometidos, como é o caso de transplantados, infetados pelo vírus da imunodeficiência humana (HIV) e aqueles com um sistema imunológico imaturo, como fetos e recém-nascidos. O HCMV também é considerado um dos mais bem-sucedidos parasitas, pois pode ser encontrado tanto em sociedades industrializadas e desenvolvidas como em grupos indígenas isolados, sendo a infeção por este vírus relativamente comum entre mulheres em idade reprodutiva, com seroprevalência variando de 45 a 100%.

O diagnóstico da infeção por HCMV permanece controverso, pois é difícil separar os pacientes assintomáticos (mas que excretam HCMV em fluidos corporais) e que poderão vir a necessitar de terapia, de pacientes com doença sintomática (pneumonia ou retinite). Atualmente, os métodos laboratoriais para o diagnóstico da infeção por HCMV podem ser divididos em técnicas sorológicas e virológicas. Os métodos sorológicos são usados principalmente para avaliar os anticorpos do doador ou do recetor em situações de transplante e prever o risco de os pacientes imunocomprometidos virem a desenvolver doença sintomática. Por outro lado, o diagnóstico virológico da doença por HCMV é geralmente baseado no isolamento do vírus por métodos de cultura. Estes métodos podem ser usados mediante a utilização de amostras de sangue, urina, saliva, fezes, lágrimas, leite materno, secreções cervicais e vaginais e sêmen. Os métodos mais comuns para o diagnóstico da infeção por HCMV são então: - testes sorológicos baseados na deteção de IgM e IgG; - a deteção direta de HCMV através de isolamento viral em cultura de fibroblastos e deteção de antigénios virais em amostras de tecido, urina ou saliva; e - PCR, que se baseia na amplificação de fragmentos específicos do genoma do HCMV e sua posterior hibridização. No entanto, estes métodos apresentam alguns inconvenientes na sua aplicação como métodos de triagem em laboratórios

de análises clínicas, pois requerem um longo período de tempo até à obtenção de um diagnóstico ou são caros. Assim, existe a necessidade de desenvolver um método que seja rápido, eficaz e barato para o diagnóstico deste vírus, capaz de ser usado em série.

Nos últimos anos, os biossensores eletroquímicos foram amplamente utilizados na determinação de variadas substâncias com diferentes propriedades e para a monitorização contínua de processos biológicos. A deteção eletroquímica é usada devido a sua sensibilidade aprimorada e custos de instrumentação reduzidos em comparação com outros métodos de transdução. Para além disto, para desenvolver dispositivos eletroquímicos confiáveis, miniaturizados e gerenciáveis, a tecnologia *screen-printing* é uma escolha inteligente. Os elétrodos serigrafados (SPE) contribuem para o desenvolvimento de novos biosensores em dispositivos miniaturizados, que apresentam as vantagens acima descritas, permitindo a obtenção de resultados em poucos minutos. Adicionalmente, os SPEs permitem uma produção massiva de sistemas eletródicos com tamanho e geometria uniformes, garantindo reprodutibilidade entre medições a baixo custo. Outra mais-valia destes sensores é o facto de serem descartáveis, o que evita alguns problemas frequentemente associados aos elétrodos tradicionais, como a necessidade de um processo de limpeza. Eles são igualmente bastante versáteis, uma vez que uma ampla gama de *designs* e materiais podem ser aplicados para na sua construção.

Na literatura podemos encontrar relatos do uso de dispositivos de deteção miniaturizados para o reconhecimento eletroquímico de sequências amplificadas de ADN provenientes de HCMV. Num desses trabalhos, baseado em elétrodos serigrafados, o ADN alvo foi adsorvido e hibridado com uma sonda de ADN biotinilada e os híbridos formados foram determinados com estreptavidina conjugada com peroxidase de rábano (HRP). Apesar da amplificação de sinal ter sido conseguida, a atividade do conjugado tem de ser controlada periodicamente devido à estabilidade da enzima. Para superar essa limitação, um outro grupo explorou outra estratégia recorrendo a marcação do ADN com nanopartículas de ouro. Apesar de terem tido melhores resultados, ambos os métodos descritos não descartam a utilização de PCR, o que os torna dispendiosos e inúteis como métodos de triagem. Um sensor piezoelétrico também foi descrito para detetar a glicoproteína do HCMV. Embora a técnica não dependa de ADN amplificado, requer o uso de instrumentação cara. Adicionalmente, um dispositivo de deteção baseado em imunofluorescência foi desenvolvido por outro grupo, aqui a amostra biológica é aplicada sobre uma superfície de ouro revestida com anticorpos específicos para HCMV (se presente em amostras biológicas, o HCMV é aprisionado na superfície deste). Ensaios positivos e negativos eram discriminados pelo uso de uma sonda fluorescente. A principal desvantagem deste dispositivo é a baixa sensibilidade que compromete a sua aplicabilidade em amostras com baixas cargas virais. Recentemente, foi ainda proposto um imunoensaio para a deteção do antígeno pp65 do HCMV utilizando HPR e nanopartículas de Pt-Pd funcionalizadas com *single-walled nanohorns* de carbono. A abordagem permitiu a deteção rápida de HCMV, no entanto, o uso de elétrodos de carbono vítreo não é uma alternativa prática para um método de triagem.

O presente trabalho descreve o desenvolvimento de um método alternativo para a deteção e quantificação de HCMV gB. O objetivo é construir um imunossensor que determine a presença de gB em amostras de urina. O uso de anticorpos de captura contra as glicoproteínas do envelope do HCMV abre a possibilidade para o desenvolvimento de novos métodos de análise imunoquímica. A glicoproteína B do HCMV (gB) é uma glicoproteína viral que desempenha um papel crucial na entrada do vírus na célula e surge durante os estágios iniciais de uma infeção pelo mesmo vírus. A gB também é o antigénio dominante presente no envelope do HCMV, sendo possível a sua determinação em fluídos corporais como a urina e saliva, onde as cargas virais são maiores. Como consequência, o desenvolvimento de novos métodos baseados na deteção de gB em fluídos corporais é de grande interesse. Para a construção dos dispositivos, usamos sempre imunoensaios com configuração em *sandwich*, pois a gB é colocada entre um anticorpo primário, previamente imobilizado numa superfície sólida, e um anticorpo secundário marcado. Os imunoensaios em *sandwich* são atualmente os mais frequentemente usados, principalmente devido a sua alta sensibilidade e correspondente minimização de interferências. Para além disto, podem ser realizados em qualquer tipo de superfície, sendo o principal critério destes ensaios a disponibilidade de dois anticorpos com sítios de ligação diferentes para o mesmo antigénio-alvo.

Durante o decorrer deste trabalho foram desenvolvidos três imunoensaios diferentes. O primeiro foi um imunoensaio eletroquímico. Foram usados anticorpos de captura anti-gB absorvidos em elétrodos de carbono serigrafados e um anticorpo secundário anti-gB marcado com nanopartículas de ouro. A deteção de gB foi realizada por meio da análise eletroquímica de nanopartículas de prata depositadas quantitativamente no imunossensor através de catálise por nanopartículas de ouro, as quais foram utilizadas como marcadores do anticorpo secundário. A reprodutibilidade do método (RSDs de cerca de 12%) não foi muito boa devido à imobilização aleatória do anticorpo primário no elétrodo de trabalho, o que resultou numa pequena eficiência de deteção do antígeno (foram observados baixos sinais considerando a grande quantidade de anticorpo utilizado). Contribui-o também para a baixa RSD observada a deposição não específica de prata na superfície do sensor. Por estas razões, decidiu-se desenvolver outra abordagem para superar as limitações observadas.

Desenvolvemos um imunoensaio enzimático espectrofotométrico baseados em partículas magnéticas (mpEIA). O uso de esferas magnéticas (MBs) funcionalizadas com proteína G (MBs-prG) como superfície sólida para a imobilização do anticorpo primário (mAb1) permite a sua fixação orientada, resultando num reconhecimento mais efetivo do gB. Para além disto, estas partículas melhoram a interação de afinidade graças a uma cinética de análise mais rápida. O anticorpo secundário foi marcado com HRP para possibilitar a deteção espectrofotométrica. Os resultados obtidos com este mpEIA espectrofotométrico são favoravelmente comparáveis com outros relatos de deteção de gB em termos de desempenho analítico. No entanto, apesar das vantagens, os leitores ELISA não podem ser aplicados como dispositivos portáteis para fazer medições *in situ*.

Para superar essa limitação, o método mpEIA mencionado acima foi adaptado à transdução eletroquímica recorrendo ao uso de elétrodos serigrafados. Esta variação visou a obtenção de um dispositivo simples, sensível, descartável e portátil. É mantido o esquema de imunoensaio com base na proteína analítica gB intercalada entre um anticorpo monoclonal primário e o anticorpo secundário anti-gB marcado com HRP, que permite igualmente deteção eletroquímica. Da mesma forma, partículas magnéticas funcionalizadas com proteína G (MBs-prG) são usadas para permitir a imobilização orientada ao anticorpo (mAb1). O imunossensor desenvolvido mostrou ser um método portátil, rápido, preciso, rigoroso, de baixo custo e, portanto, eficaz na deteção de gB em amostras de urina humana para a valiosa triagem de infeções por HCMV.

Palavras-chave

Citomegalovírus humano, glicoproteína B, imunoensaios, mpEIA, imunossensores eletroquímicos, elétrodos serigrafados

Abstract

Human Cytomegalovirus (HCMV) is a herpes virus that establish a lifelong latent infection of the host, so once a person is infected, the virus persists in a state of cellular latency. Following primary infection, HCMV is excreted in body fluids and its transmission occurs through mucous contact and exposure to urine, blood transfusion and organ or bone marrow transplant procedures, being extremely difficult to identify the transmission route.

HCMV infection induces no overt disease in healthy carriers, owing to effective immune control, but this infection can be severe or even fatal in immunosuppressed individuals, fetuses and newborns. Furthermore, HCMV is also relatively common among women in reproductive age, with seroprevalence ranging from 45 to 100%.

The diagnosis of HCMV disease remains controversial because of the difficulty of separating patients who are asymptomatic but shedding HCMV in body fluids, from patients who have the symptomatic disease. Nowadays the most common methods for diagnosis of HCMV infection are: - serological tests based on IgM and IgG detection; - direct free HCMV detection by viral isolation and viral antigens detection in tissue, urine or saliva samples; and - PCR, which is based on amplification of selected segments of the HCMV genome and its hybridization. However, these methods are disadvantageous to be routinely used in clinical diagnosis as point of care because they require a long time to perform or are costly. Thus, there is a need to develop a method which is fast, effective and inexpensive for this virus diagnosis.

As an alternative, the use of capture antibodies against the envelope glycoproteins of HCMV open the possibility of faster immunochemical methods. Glycoprotein B of HCMV (gB) is the dominant antigen in the envelope of HCMV, being possible its determination in body fluids like urine and saliva, where viral loads are higher. In consequence, the development of new methods based on the accurate detection of gB in body fluids, is of great interest.

In recent years, electrochemical biosensors were widely used to determine various substances with different properties and for continuous monitoring of biological processes. Bioanalytical assays such as immunoassays (IAs), are also very important in many fields. IAs are based on antibodies ability to form complexes with the corresponding antigen, making them highly specific and selective. Thus, electrochemical immunoassays offer enhanced sensitivities and reduced instrumentation costs compared to their counterparts using other transducing elements. Also, screen-printed electrodes (SPE) contribute to develop miniaturized, easy to handle and reliable IAs devices. In addition, SPEs allow for a high-volume production of electrode systems with uniform size and geometry, ensuring measurement reproducibility at low cost. They are also very versatile, since a wide range of designs and materials can be applied in their construction.

The present work describes the development of an alternative method for HCMV gB detection and quantification. It is intended the development of an immunosensor to quantify the presence of gB in urine samples. For the construction of this device we made use of a

sandwich type immunoassay, wherein HCMV gB is sandwiched between a primary antibody, previously immobilized on a solid surface, and a labelled secondary antibody. Sandwich immunoassays are currently the most commonly and successfully used, mainly due to their high sensitivity and minimized background signal. Moreover, they can be performed on any kind of sensing surface, being the main criterion for these assays the availability of two antibodies with different binding sites on the target antigens.

Three different immunoassays were developed. The first one was an electrochemical immunoassay, gB detection was carried out over electrochemical stripping analysis of silver nanoparticles quantitatively deposited on the immunosensor through catalysis by nanogold labels. Capture anti-gB antibodies were absorbed on screen-printed carbon electrodes, and a secondary anti-gB antibody labelled with gold nanoparticles. Nevertheless, the reproducibility of the method (RSDs ≈ 12%) was not very good owing to the random immobilization of the primary antibody on the working electrode, which resulted in small efficiency of antigen detection. Contributing to the low observed RSD was also the nonspecific deposition of silver on the sensor surface. For these reasons, it was decided the development of another approach to overcome the observed limitations. A spectrophotometric magnetic particle-based enzyme immunoassays (mpEIA) was constructed. The use of magnetic beads (MBs) functionalized with protein G (MBs-prG) as solid surface for primary antibody (mAb1) immobilization allows its oriented attachment, resulting in a more effective recognition of gB. Additionally, they improve the affinity interaction thanks to a faster assay kinetics of the dispersed beads in urine samples. The results obtained with this spectrophotometric mpEIA compared favorably to those obtained in other reports of gB detection in terms of analytical performance. Despite the advantages, ELISA readers cannot be applied as portable devices to make in situ measurements. It was then proposed an adaptation to electrochemical transduction on screen-printed electrodes. This variation aimed the achievement of a simple, sensitive, disposable and portable device. It was maintained the immunoassay scheme based on the analyte protein gB sandwiched between the primary monoclonal antibody and the secondary anti-gB-HCMV HRP labelled antibody. Similarly, magnetic particles functionalized with protein G (MBs-prG), were used.

The developed immunosensor was shown to be a portable, fast, accurate, rigorous, low cost and an effective method of detecting gB in human urine samples for the valuable diagnosis/screening of HCMV infections.

Keywords

Human cytomegalovirus, glycoprotein B, immunoassays, mpEIA, electrochemical immunosensors, screen printed electrodes.

Thesis Overview

This doctoral thesis is structured in five chapters appendices, herein summarized:

CHAPTER I consists on a brief introduction aiming literature review and the definition of PhD thesis objectives. State of the art description emphases:

- Topics on human cytomegalovirus, with focus on virus structure as well as the problems associated to viral infection in humans;
- Basics underlining the construction of an immunoassay along with immunoassay advantages and disadvantages;
- Fundamentals on the construction of an electrochemical biosensor. With focus on the steps required for its assembly, from the choice of the biological recognition system to the choice of the transducer. Praticular importance was given to electrochemical immunosensors, once they represent the main objective of this work.

CHAPTER II discusses and integrates all the results obtained for the development of a sandwich type immunosensor for the detection of human cytomegalovirus glycoprotein B through electrochemical stripping analysis of silver nanoparticles quantitatively deposited on the immunosensor through catalysis by nanogold labels.

CHAPTER III discusses and integrates all the results obtained for the development of a magnetic particle-based enzyme immunoassay for human cytomegalovirus glycoprotein quantification

CHAPTER IV discusses and integrates all the results obtained for the development of an amperometric immunosensor for Human cytomegalovirus glycoprotein B detection

CHAPTER V presents the conclusions of this thesis and future remarks

APPENDIX consists on the presentation of the electrochemical immunosensors national patent granted during the course of this work

List of Publications

F. Pires, J. C. Vidal, J. R. Castillo, M. J. Arcos-Martínez, A. C. Dias-Cabral. Magnetic particles-based amperometric immunosensor for Human Cytomegalovirus glycoprotein B detection. (paper submitted to Talanta)

F. Pires, A. C. Dias-Cabral, M. J. Arcos-Martínez, J. C. Vidal, J. R. Castillo. A rapid magnetic particle-based enzyme immunoassay for human cytomegalovirus glycoprotein B quantification. Journal of Pharmaceutical and Biomedical Analysis 165 (2018): 372-378.

F. Pires, Hugo Silva, O. Domínguez-Renedo, M. A. Alonso-Lomillo, M. J. Arcos-Martínez, A. C. Dias-Cabral. Disposable immunosensor for human cytomegalovirus glycoprotein B detection. Talanta 136 (2015): 42-46.

F. Pires, F. Marques, S. Almeida, F. C. Domingues, O. Domínguez-Renedo, M. A. Alonso-Lomillo, M.J. Arcos Martínez, A. C. Dias-Cabral. "Dispositivo electródico para deteção de glicoproteína B, procedimento de fabrico e uso dos ditos dispositivos". National invention patent nº 107029. Published in Boletim de Propriedade Industrial nº 248/2014 edited on 2014-12029. Patente granted on 2015 - 07- 08.

Oral communications presented and prizes obtained during the PhD Course

J. Carvalho, S. Miguel, A. Moreira, J. Boga, **F. Pires**. Viruscan-sensors for viral detection. SCIENT Entrepreneurship Academy and Business Competition, Nicosia, Cyprus, September, 2017 (First prize).

F. Pires, M.J. Arcos-Martínez; A.C. Dias-Cabral, J.C. Vidal, J.R. Castillo, "mpEIA for Human Cytomegalovirus glycoprotein B detection". XII annual CICS-UBI symposium. Covilhã, Portugal, July 2017 (award of best PhD oral communication).

F. Pires, J.C. Vidal, J.R. Castillo, M.J. Arcos-Martínez; A.C. Dias-Cabral; "Magneticparticles-based enzyme immunoassay for Human Cytomegalovirus detection". II International Congress in Health Sciences Research. Covilhã, Portugal, May 2017.

F. Pires, J.C. Vidal, J.R. Castillo, M.J. Arcos-Martínez, A.C. Dias-Cabral. "New spectrophotometric immunosensor for human cytomegalovirus glycoprotein B detection". XI Annual CICS Symposium. Covilhã, Portugal. July 2016.

F. Pires, H.Silva, O. Domínguez-Renedo, M. A. Alonso-Lomillo, M.J. Arcos Martínez, A. C. Dias-Cabral. "Disposable immunosensor for human cytomegalovirus glycoprotein B detection". XXXVI Reunión del grupo de electroquímica de la Real Sociedad Española de Química XVII Encontro Ibérico de Electroquímica. Vigo, Spain. July 2015

F. Pires, H.Silva, O. Domínguez-Renedo, M. A. Alonso-Lomillo, M.J. Arcos Martínez, A. C. Dias-Cabral. "Imunossensor descartável para a detecção de glicoproteína B do citomegalovírus humano". 4º Ciclo de Conferências da Faculdade de Ciências da Universidade da Beira Interior. Covilhã, Portugal. January 2015

F. Pires, H.Silva, O. Domínguez-Renedo, M. A. Alonso-Lomillo, M.J. Arcos Martínez, A. C. Dias-Cabral. "Detection of Human Cytomegalovirus glycoprotein B using a disposable immunosensor". IX Annual CICS Symposium. Covilhã, Portugal. July 2014

Poster communications presented during the PhD Course

F. Pires, J. C. Vidal, J. R. Castillo, M. J. Arcos-Martínez, A.C Dias-Cabral. "New immunosensor for human Cytomegalovirus detection". Encontro Ciência, Lisbon, Portugal, July 2016.

F. Pires, J.C. Vidal, J.R. Castillo, M.J. Arcos-Martínez A.C. Dias-Cabral. "New Amperometric immunosensors for Human Cytomegalovirus glycoprotein B detection". BioIberoAmerica 2016. Salamanca, Spain, June 2016.

F. Pires, J.C. Vidal, J.R. Castillo, M.J. Arcos-Martínez, A.C. Dias-Cabral. "Nuevos inmunosensores amperométricos para la determinación de la glicoproteína B del citomegalovirus humano". IV Jornadas del IUCA. Zaragoza, Spain, November 2015.

F.Pires, O. Domíngues-Renedo, M.A. Alonso-Lomillo, M.J.Arcos Martínez, A.C. Dias-Cabral. "Development of an immunosensor for human cytomegalovirus glycoprotein b detection. X annual CICS-UBI symposium. Covilhã, Portugal, July 2015.

F. Pires, H. Silva, O. Dominguez-Renedo, M.A. Alonso-Lomillo, A.C. Dias-Cabral, M.J. Arcos-Martínez. "Studies towards the development of a disposable immunosensor for diagnosis of Human Cytomegalovirus congenital infection- immobilization methods". 10th European Symposium on Biochemical Engineering Sciences and 6th International Forum on Industrial Bioprocesses in collaboration with ACS. Lille, France, September 2014.

F. Pires, H.Silva, O. Domínguez-Renedo, M. A. Alonso-Lomillo, A. C. Dias-Cabral, M.J. Arcos Martínez. "Disposable Immunosensor for diagnosis of Human Cytomegalovirus congenital infection". XXXV Meeting of Electrochemistry of the Spanish Royal Society of Chemistry e 1st E3 Mediterranean Symposium: Electrochemistry for Environment and Energy. Burgos, Spain, July 2014.

F.Pires, O. Domíngues-Renedo, M.A. Alonso-Lomillo, M.J.Arcos Martínez, A.C. Dias-Cabral. "Disposable Immunosensor for diagnosis of Human Cytomegalovirus congenital infection". MicroBiotec. Aveiro, Portugal, December 2013.

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List of abbreviations

A

aa	Aminoacid
Ab	Antibody
Ab2-HRP	Antibody labelled with *Horseradish peroxidase*
Ab-AuNPs	Antibody labelled with gold nanoparticles
ABS	Antigen binding sites
AD	Antigenic domain
AFM	Atomic force microscopy
Ag	Antigen
AgNPs	Silver nanoparticles
AuNPs	Gold nanoparticles

B

BTM	Bone marrow transplantation
BSA	Bovine serum albumin

C

C	Antibody constant region
CE	Counter electrode
C_H	Heavy constant region
C_L	Light constant region
CDR	Complementarity-determining region
CNS	Central nervous system
CNTs	Carbon nanotubes
CTL	Cytotoxic T lymphocytes
CVC	Capsid vertex-capping

D

DPI	Dual polarization interferometry

E

E	Early

EBV	Epstein Barr vírus
ELISA	Enzyme-linked immunosorbent assay
EMEA	European Medicines Agency
ERGIC	Endoplasmic reticulum and Golgi complex intermediate compartment

F

Fab	Antigen-binding fragments
FTIR	Fourier transform infrared reflection

G

gB	Glycoprotein B
gH	Glycoprotein H
gL	Glycoprotein L
gM	Glycoprotein M
gM	Glycoprotein M
gO	Glycoprotein O

H

HCMV	Human cytomegalovirus
HDAC	Histone deacetylase
HHV	Human herpes viruses
HIV	Human immunodeficiency virus
HRP	*Horseradish peroxidase*
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2

I

IAs	Immunoassays
IE	Immediate-early
IF	Immunofluorescence
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ITAMS	Immunoreceptor tyrosine-based activation motifs

K

KSHV	Kaposi's sarcoma associated herpes

L

L	Late
LOD	Limit of detection
LTP	Large tegument protein
LTPbp	Large tegument binding protein

M

mAb	Monoclonal antibody
mAb1	Primary monoclonal antibody
MBs	Magnetic micro-beads
MBs-PrG	Magnetic particles functionalized with protein G
MBs-PrG-mAb1	Complex of magnetic particles functionalized with protein G with primary monoclonal antibody
mCBP	Minor capsid binding protein
MCP	Major capsid protein
mCP	Minor capsid protein
MIE	Major immediate early
mpEIA	Magnetic particle-based enzyme immunoassay
MWCNT	Multi-wall carbon-nanotubes

N

NHS	N-hydroxysuccinimide
NK	Natural killer
NMNPs	Noble metal nanoparticles
NPs	Nanoparticles
NR	Neutron reflectometry
NSA	Non-specific adsorptions

O

ORF	Open Reading frame
Ori Lyt	Origin of DNA synthesis

P

pAb	Polyclonal antibody
pAP	Assembly protein precursor
PBL	Peripheral blood leukocytes
PCR	Polymerase chain reaction
pMHC	Peptide-major histocompatibility complexes
pNP1	Proteinase precursor
PoC	Point of care
PORT	Portal protein

Q

QD	Quantum dots

R

RE	Reference electrode
RIA	Radioimmunoassay
RSD	Relative standard deviation
RT-PCR	Reverse transcriptase polymerase chain reaction

S

SAXs	Small-angle X-ray scattering
SCP	Small capsid protein
SPE	Screen printed electrode
SPR	Surface plasmon resonance
SU	Surface subunit
SWCNT	Single-wall carbon-nanotubes

T

TCR	T cell antigen receptors
TER1	Terminase subunit 1
TER2	Terminase subunit 1
TM	Transmembrane
TMB	Peroxidase substrate 3,3',5,5'-tetramethylbenzidine liquid substrate system for ELISA
ToF-SIMS	Time-of-flight secondary ion mass spectrometry

V

V	Antibody variable region
V_H	Heavy variable region
V_L	Light variable region
VZV	Varicella-zoster virus

W

WE	Working electrode

ß

ßgal	*ß-galactosidase*

CHAPTER I

General Introduction

I. 1. Human Cytomegalovirus

I.1.1 Human Cytomegalovirus Overview

Human Cytomegalovirus (HCMV) is the most usual name for herpesvirus 5, a virus that belongs to *Herpesviridae* family and *β-herpesviridae* subfamily [1]. It is the largest virus of the family, with 200 nm in diameter, 240 kb in size and a molecular weight of 155 kDa, and is morphological indistinguishable from other herpes viruses, with a linear double-stranded DNA genome packaged in an icosahedral capsid (figure I.1). The capsid is surrounded by a protein layer known as tegument, and this is enclosed in a lipid bilayer that contains 6 encoded glycoproteins, gpUL55 (gB, glycoprotein B), gpUL73 (gN, glycoprotein N), gpUL74 (gO, glycoprotein O), gpUL75 (gH, glycoprotein H), UL100 (gM, glycoprotein M) and gpUL115(gL, glycoprotein L) [1-5]. These glycoproteins perform an important role in the initial process of interaction with the host cell [3,5]. Specialy, glycoprotein B plays a crucial role in virus binding, entry, cell-to-cell spread and cell fusion [5]. In addition, gB is the major antigen, capable to induce neutralizing antibodies against HCMV [3].

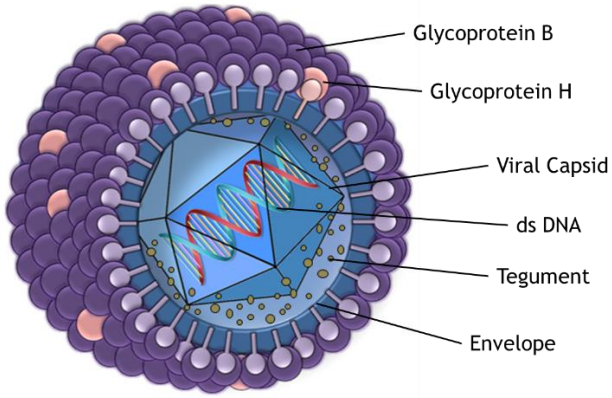

Figure I. 1. Representation of Human Cytomegalovirus (HCMV).

As for other herpesviruses, the assembly of the infectious particle is a complex and poorly understood process [2].

Human being is the only known receptor for HCMV and its transmission can occur both vertically and horizontally. HCMV is one of the most successful parasites, it can be found in both developed industrial societies and in isolated aboriginal groups, being relatively common among women in reproductive age, with seroprevalence ranging from 45 to 100% [5,6].

HCMV is considered a virus of paradoxes, because it can be a potential killer or a lifelong silent companion. The infection by HCMV results in the establishment of a lifelong latent infection of the host, so once a person is infected, the virus persists in a state of cellular latency, in which infected cells are not producing any infectious virus, but retain the complete genome and have the potential to start producing virus at a later time [1,3,4,7]. HCMV infection can emerge following primary infection, reinfection or reactivation [5]. In healthy carriers it

induces no overt disease, due to effective immune control, but the infection can be severe and even fatal in immunosuppressed individuals, as transplanted ones, persons infected by human immunodeficiency virus (HIV) and those with an immature immune system, like fetuses and newborns [7,8]. Nevertheless, reactivation from latency to a state of active replication, is the major cause of disease and can occur in a situation of immune system dysfunction [3,4].

In the case of immunosuppressed individuals infected by HCMV, pneumonia, retinitis, colitis and encephalopathies may be diagnosed, while newborns can manifest microcephaly, small body size, hepatomegaly, blindness, deafness, mental retardation, among other pathologies [9]. Moreover, the infection by HCMV is the most frequent cause of embryonic and fetal pathology induced by a virus in the whole world, although the majority of the infected children does not manifest any symptom at birth [5,9]. In newborns, HCMV may be acquired in utero via placenta or by exposure to maternal genital tract during labor. In this case, most perinatal infection by HCMV are asymptomatic[5] and severe disease acquired perinatally occurs in most of the cases in child underweight [9,10].

Primary infection does not usually result in a clinical illness, except in cases of congenital infection [5,7]. Nevertheless, reactivation from latency to a state of active replication, is the major cause of disease and can occur in a situation of immune system dysfunction, which can results from other illnesses [3,4].

In immunocompromised patients HCMV infection is mostly controlled by available antiviral drugs, yet it continues to maintain its role as one of the most dangerous infectious agent for the unborn infant [9].

I.1.2 The Herpesviridae family

Herpesviridae is a family of large and complex ubiquitous viruses that are constituted by three major structural regions, capsid, tegument, and a lipid-containing envelope [2,11]. Aside from a similar morphology and some similarities in replication cycles, the major feature shared by all herpesviruses is the capacity to establish latent infection. Latent infection is defined as a type of persistent infection in which the viral genome is present but there is no production of infectious virus, except during intermittent episodes of reactivation [1,12-16]. Following initial infection, the viruses can be reactivated from the latent state producing, occasionally, episodes of significant, or serious disease. In this way, latency is the central feature of herpesviruses, however, the basic mechanisms involved in the processes of establishment and maintenance of latency and reactivation are not totally understood[2,16,17].

Eight human herpesviruses (HHV) are known. These viruses are morphologically identical (figure I.1.), with a virion consisting of an icosahedral nucleocapsid of about 100 nm of diameter, surrounded by a lipid bilayer envelope. Between the capsid and the envelope exists an amorphous layer of proteins, termed the tegument [17].

HHV are divided into the α, β, and γ-herpesvirus subfamilies (Table I.1.) and this division was based on shared biological properties (i.e., host range, replication kinetics, and ability to spread in culture) and genetic relatedness [11].

The α herpesvirus subfamily consists of herpes simplex viruses types 1 and 2 (HSV-1 and HSV-2, respectively) and varicella-zoster virus (VZV). These HHV have relatively short reproductive cycles (measured in days) and cause cytopathic effects on infected cells. They establish latent infections predominantly in the sensory ganglia[17]. Human cytomegalovirus (HCMV), HHV-6A and B and HHV-7 make part of β-herpesviruses. These viruses have longer reproductive cycles (about weeks) and the infected cells often become enlarged. During latency period they can be maintained in leukocytes, kidneys, secretory glands and other tissues. Finally, γ- herpesviruses are Epstein-Barr virus (EBV) and HHV-8. This subfamily of herpesviruses is tropic for either T- or B-lymphocytes, and latency is often established in lymphoid tissue [17].

Herpes simplex viruses types 1 and 2 (HSV-1 and HSV-2) are among the most common human infectious viral pathogens [15]. Primary HSV-1 and HSV-2 infection usually involve ectodermally derived tissues, being the mucosal membranes of the mouth, throat, genitals and corneal epithelium the most affected tissiues [17]. As the site of infection is often the site at which the lesions appear, HSV clinical manifestation can be cold sores, genital ulcerations and corneal blindness [15-17]. After initial infection, virus (or at least a subviral particle with an associated viral genome) enter in the peripheral sensory nerves and migrate along nerve axons to associated in sensory ganglia [16].Upon reactivation from the latent state, the viruses migrate back along sensory nerves to the body surface [17].

VZV is a ubiquitous virus, which causes varicella (chicken pox) and herpes zoster (shingles). While varicella results from primary VZV infection, and it is a common childhood illness associated with fever and a generalized pruritic vesicular, herpes zoster is caused by VZV reactivation. Herpes zoster is characterized by a localized, painful and vesicular rash involving one or adjacent dermatomes. The incidence of herpes zoster increases with age or immunosuppression [12]. After initial replication in the respiratory epithelium, virus spreads to regional lymph nodes and to the liver and spleen. Secondary viremia is mediated by mononuclear cells, which transport infection to cutaneous epithelial cells and respiratory mucosa. During this process, vesicles appear (chickenpox). VZV persists in the sensory ganglia of the central nervous system (CNS) [17].

HCMV is one of the most common cause of congenital infection in humans [18]. During the acute infection, epithelium-derived cells (e.g. ductal cells in the kidneys, alveolar cells in the lungs, and hepatocytes) are most commonly involved in viral replication. There is also evidence for leukocyte involvement during acute disease, with mononuclear cells being the most prominent ones [16].

HHV-6 has two distinct subtypes: HHV-6A and HHV-6B, being A variant more common[14]. Primary infection occurs commonly in early childhood. *Exanthema subitum* (*roseola infantum*) is caused by HHV-6B, whereas disease associations for HHV-6A remain ambiguous. Both HHV-6A

and HHV-6B are lymphotropic [17]. HHV-7 is similar to HHV-6A and -6B in many ways (for example, its genome sequence and biology and it may also cause exanthema subitum) [17]. This virus was initially isolated from CD4+ T cells of a healthy individual when the activated cells in culture showed cytopathic effects. It can be detected in peripheral blood [14]. Nevertheless, little is known about HHV-7 [17].

EBV virus infection results in clinical manifestation that include infectious mononucleosis, fever, sore throat, cervical and generalized lymphadenopathy, hepatosplenomegaly, and somatic complaints of fatigue and malaise [13]. EBV is transmitted through saliva, specially through the handling of toys, in the case of toddlers, or by kissing, being this the reason why infectious mononucleosis is commonly called "kissing disease" [13]. During latent stage, virus is maintained in B-lymphocytes [16].

HHV-8 or Kaposi's sarcoma associated herpes virus (KSHV) is the most recent addition to the HHV family [17]. HHV-8 is detectable in Kaposi's sarcoma lesions and may also be associated with other malignancies, since it seems that infect lymphocytes and is associated with cell immortalization and transformation [14,17].

Table I. 1. Human herpesviruses basic properties. Adapted from Ref.[17].

		COMMON NAME	GENOME SIZE	PRIMARY TARGET	SITE OF LATENCY
Human α-herpesviruses	HHV-1	Herpes simplex virus type 1 (HSV-1)	≈152 kb	Mucoepothelial cells (predominantly orofacial tract)	Neurons
	HHV-2	Herpes simplex virus type 2 (HSV-2)	≈154 kb	Mucoepothelial cells (predominantly genital tract)	Neurons
	HHV-3	Varicella zoster virus (VZV)	≈125 kb	Respiratory epithelium	Neurons
Human ß-herpesviruses	HHV-5	Human cytomegalovirus (HCMV)	≈240 kb	Epithelial cells, monocytes, fibroblasts and more.	Leukocytes, epithelial cells
	HHV-6 (A and B)	Roseleovirus	≈160 kb	Epithelial cells, monocytes, fibroblasts and more.	Leukocytes, epithelial cells
	HHV-7	Roseleovirus	≈145 kb	Epithelial cells, monocytes, fibroblasts and more.	Leukocytes, epithelial cells
Human γ-herpesviruses	HHV-4	Epstein Barr virus (EBV)	≈184 kb	T- or B-lymphocytes	Lymphoid tissue B cells
	HHV-8	Kaposi's sarcoma associated herpes virus (KSHV)	≈170 kb	Uncertain	Uncertain

I.1.3. About the Human Cytomegalovirus

I.1.3.1. Structure

HCMV virion appears to be structurally similar to those of other herpesviruses, with a DNA core inside of a icosahedral capsid made up of 162 capsomeres surrounded by an envelope derived from host cell membrane containing viral glycoproteins to control attachment and entry into cells[19]. As the larger virus of the family, HCMV has an icosahedral nucleocapsid with a diameter of 100 nm that accommodates a 240 kb double stranded linear DNA genome. The nucleocapsid of HCMV is surrounded by protein layers, called tegument that, in its turn, is enclosed by an lipidic bilayer (envelope) that harbor a large number of viral glycoproteins [1-4]. Overall, the HCM virion is the most structurally complex of the herpesviruses and the mature virion presents a diameter of 200 nm, approximately [3,19].

HCMV capsids are composed of four core proteins: major capsid protein (MCP), encoded by UL86, minor capsid protein (mCP), encoded by UL85, minor capsid binding protein (mCBP) encoded by UL46 and the smallest capsid protein (SCP) encoded by UL48.5 [20] (Table I.2). Organization of these four proteins into a capsid appears to be coordinated by two genetically related, internally situated proteins, called the proteinase precursor (pNP1), encoded by UL80a, and the assembly protein precursor (pAP), encoded by UL80.5 [20]. One specialized penton composed of the portal protein (PORT), encoded by UL104 acts as a channel for both encapsidation and release of viral DNA together with two principal subunits of the terminase, subunit 1 (TER1), encoded by UL89 gene and subunit 2 (TER2), encoded by the UL56. A capsid vertex-capping (CVC) complex composed of UL77 and UL93 proteins decorates all pentons and the proteins encoded by UL51 and UL52 provide stability[20].Therefore, hexons from MCP are responsible for the triangular faces of the capsid and their conjugation with pentons from MCP and PORT form the bulk of the 15-nm–thick capsid walls[19].

The viral tegument is the region located between the capsid and the envelope and contains approximately 40% of the virion protein mass (Table I.2). Nevertheless, little is known about its structure or function [21,22]. Nowadays, at least 32 virus-encoded proteins are known on tegument, many of which are phosphorylated. These proteins, may play important roles in viral gene regulation, in modification of the host cell metabolism and in virion assembly [22]. The most abundant tegument protein in virions is pp65, encoded by UL83. This protein is highly immunogenic, however, despite its abundance and importance during natural infection, UL83 is dispensable for virus replication [19].

Virions, dense bodies and other noninfectious virus particles are enclosed in a lipid bilayer envelope. The envelope is derived from the endoplasmic reticulum, Golgi complex intermediate compartment (ERGIC) or endosomal membranes. The envelope has approximately 66 virus encoded proteins that play diverse roles during infection, ranging from mediating and

modulating entry and egress virus in the cell, influencing cell tropism, and interacting with the host response to infection. Nevertheless, just six envelope glycoproteins (gB, gH, gL, gM, gN and gO) provide essential replication functions and are targets of neutralizing antibody [19] (Table I.2).

Glycoprotein B (gB) or gpUL55 is perhaps the most highly conserved envelope component and is also the most abundant envelope protein. gB function in the replicative cycle of HCMV is undefined, but studies suggest that it participates in both attachment and fusion of the virion to the cell [23]. The characteristics and role of this protein will be discussed with more detail in section I.1.3.1.1

Glycoprotein H (gH) or gpUL75 is lesser abundant than gB, however is perhaps the next most abundant protein component in the envelope. It has been proposed that gH mediates viral/host cell membrane fusion in the initial steps of virus infectivity. Also, monoclonal anti-gH antibodies exhibit potent virus neutralizing activity. This glycoprotein has been reported to associate covalently with glycoprotein L (gL) or gpUL100 and glycoprotein O (gO) or gpUL74 [23]. gH/gL/gO complex mediate virus replication, more specifically its entry into epithelial and endothelial cells, but its basic structure is uncharacterized [24,25]

Glycoprotein M (gM) or gpUL100 is also important during virus fusion and/entry in the cell and cell-to-cell virus spread. This protein forms a complex with glycoprotein N (gN) or gpUL55 and little is known about this complex immunogenicity during natural infection, nevertheless, as envelope glycoproteins, it is believed that they equally induce neutralizing antibodies [26]. In conclusion, and as already was mentioned, these envelope glycoproteins linked covalently to associate and form complexes that are highly conserved. Presently, the HCMV envelope consists of at least three distinct glycoprotein complexes, designed gCI, gCII, gCIII [27]. gCI is composed by homodimeric gB molecules, gCII is composed by gM and gN and gCIII is a heteroligomeric complex composed of gH, gL, and gO and all of these three complexes induce neutralizing antibodies [5,25,28].

Table I. 2. Selection of expression structure HCMV proteins. Adapted from Ref. [5] and [19].

EXPRESSION STRUCTURE	PROTEIN	HCMV GENE	FUNCTION/ COMMENTS
Capsid	mCBP	UL46	Capsid component; minor capsid binding protein.
	SCP	UL 48.5	Capsid component; smallest capsid protein
	pUL51	UL51	Necessary for nucleocapsids formation and DNA encapsidation
	pUL52	UL52	Necessary for nucleocapsids formation and DNA encapsidation

Table I. 1. Selection of expression structure HCMV proteins. Adapted from [5] and [19] (continued).

EXPRESSION STRUCTURE	PROTEIN	HCMV GENE	FUNCTION/ COMMENTS
Capsid	TER2	UL56	Binds to DNA packaging motif, exhibits nuclease activity (DNA encapsidation)
	pUL77	UL77	Supposed capsid vertex component; DNA encapsidation
	pNP1	UL80A	Capsid assembly
	pAP	UL80.5	Capsid assembly
	mCP	UL85	Minor capsid protein; capsid component
	MCP	UL86	Major capsid protein; capsid component
	TER1	UL89	Inhibition by antiviral compounds, DNA encapsidation
	pUL93	UL93	Supposed capsid vertex component; DNA encapsidation
	PORT	UL104	DNA encapsidation
Tegument	pUL36	UL36 (IE)	IE; blocks apoptosis
	LTPbp	UL47	Associates with pUL48 and supports intracellular capsid transport
	LTP	UL48	Largest tegument protein, associates with pUL47 and is responsible for intracellular capsid transport
	pp65	UL83	Gene regulation
	pIRS1	IRS1 (IE)	Transactivator of viral gene expression
	pTRS1	TRS1 (IE)	Transactivator of viral gene expression
Envelope	gB	UL55	Major envelope glycoprotein, Constituent of gCI, mediator of viral entry in the cell
	gN	UL73	Constituent of gCII, complexes with gM to support envelopment
	gO	UL74	Constituent of gCIII; enhances gH-gL delivery and release of virions
	gH	UL75	Constituent of gCIII; complexed with gL and gO; role in entry in the cell

Table I. 2. HCMV proteins discussed in this work. Adapted from Ref. [5] and [19] (continued).

EXPRESSION STRUCTURE	PROTEIN	HCMV GENE	FUNCTION/ COMMENTS
Envelope	gM	UL100	Constituent of gCII; complexes with gN; role in envelopment.
	gL	UL115	Complexed with gH-gO; role in entry to the cell
Core	p52	UL44	DNA processivity factor
	DNA pol.	UL54	DNA polymerase; DNA synthesis
	pUL57, DNAss	UL57 *Ori Lyt*	DNA synthesis, single-stranded DNA-binding protein
	Helicase primase	UL70	Helicase-primase subunit
	pUL84	UL84	Initiation of oriLyt-specific DNA replication
	Helicase primase	UL102	Helicase-primase subunit
	Helicase primase	UL105	Helicase-primase subunit
	Early pp	UL112	Organization of viral DNA replication centers
	Early pp	UL113	Organization of viral DNA replication centers
	pUL114	UL114	Uracil DNA glycosylase
	IE2	UL122 (IE)	Regulation of viral and host gene expression
	IE1	UL123 (IE)	Regulation of viral and host gene expression
ER, mitochondria and membrane	vMIA	UL37 (IE)	IE; blocks apoptosis
Membrane	gpUS3	US3 (IE)	Down-modulation of MHC Class I expression

I.1.3.1.1. Glycoprotein B

Glycoprotein B (gB), as the most abundant protein in HCMV envelope, represents more than 50% of the envelope protein mass, and its encoding open reading frame (ORF), UL55, is probably the most highly conserved genome segment of the virus [23]. Additionally, it is the most easily detected envelope glycoprotein in infected cells, having, as mentioned before, an important role in replicative HCMV cycle, participating in both attachment and fusion of the virion to the cell [23,29].

gB is a type I membrane glycoprotein, being composed by a transmembrane (TM), gp55 subunit and a surface subunit (SU), gp 116 (Fig. I.2a.). The analysis of aminoacids (aa) sequence from strain AD169 has suggested the presence of cleavable signal aa sequence between 1-26 aa, and three potential hydrophobic regions. These regions are aa between 714-747, 751-771 and 784-792 (figure I.2b.). The deletion of the first hydrophobic region leads to the failure in protein secretion, resulting in a protein that is incompletely processed. The second hydrophobic region, 751-771 aa, and the most hydrophobic domain is responsible for the gB insertion in the membrane. The importance of the third hydrophobic fourth region is still undefined [23].

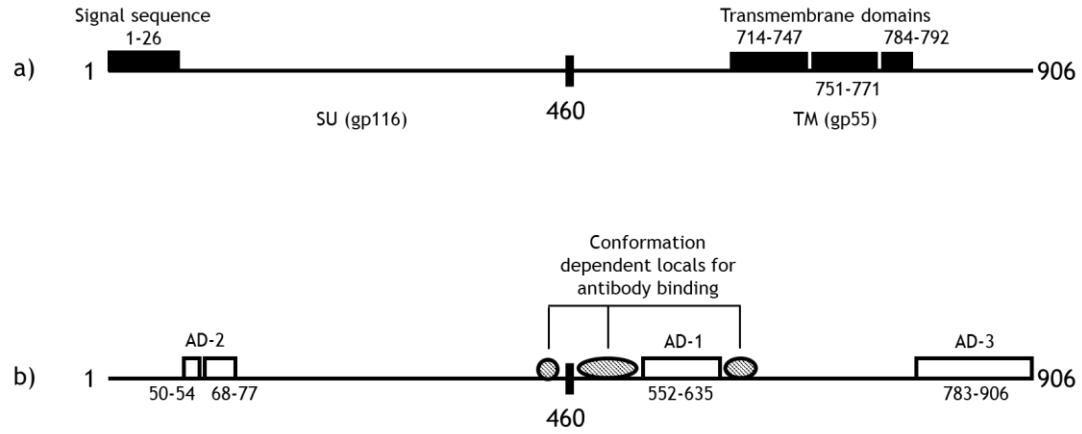

Figure I. 2. The 906 aa sequence of HCMV strain AD169 gB. a) Several structural features of gB. b) Antibody binding sites (AD-1, AD-2 and AD-3) and regions which are thought to contribute to assembled, conformational dependent antibody binding sites. Adapted from Ref. [23] and [30].

The 906 aa polypeptide gB of strain AD169 is post-translationally modified into a 160 kDa glycosylated precursor molecule which is subsequently proteolytically cleaved in the position 460. Both subunits remain covalently linked by disulphide, representing the mature intracellular as well as the virion form of gB [10].

Several studies have demonstrated that a considerable fraction of the virus-neutralizing activity found in human serum following natural infection is directed against gB, addressing to gB the role of the dominant antigen on the envelope of HCMV [10,30].

Three antibody-binding sites have been identified on gB: antigenic domain 1 (AD-1), located between aa 552-635; AD-2, aa 50–77; and AD-3, aa 783–906 (figure I.2b.) [30]. AD-1 represents the dominant antibody-binding site on gB, once nearly all of the infected individuals who are seropositive for gB have antibodies against AD-1. Antibody binding requires the entire AD-1 sequence [30]. AD-2comprises two sites, local I (residues 68-77) and local II (residues 50-54) [10]. Of these three domains, just domain AD-1 and local II of AD-2 are able to induce virus-neutralizing antibodies during the natural infection[10].

I.1.3.2. Infectious cycle

Virus entry occurs in distinct steps: (I) binding to specific cell surface receptors, (II) viral envelope fusion with cellular membranes to release nucleocapsids into the cytoplasm, either directly at the plasma membrane (as occurs in fibroblasts) or after endocytosis into cells (as occurs in endothelial and epithelial cells), (III) nucleocapsid translocation toward the nucleus on cytoskeletal filaments, (IV) nucleocapsid interaction with nuclear pores, and (V) release of the viral genome into the nucleus [19] (figure I.3.).

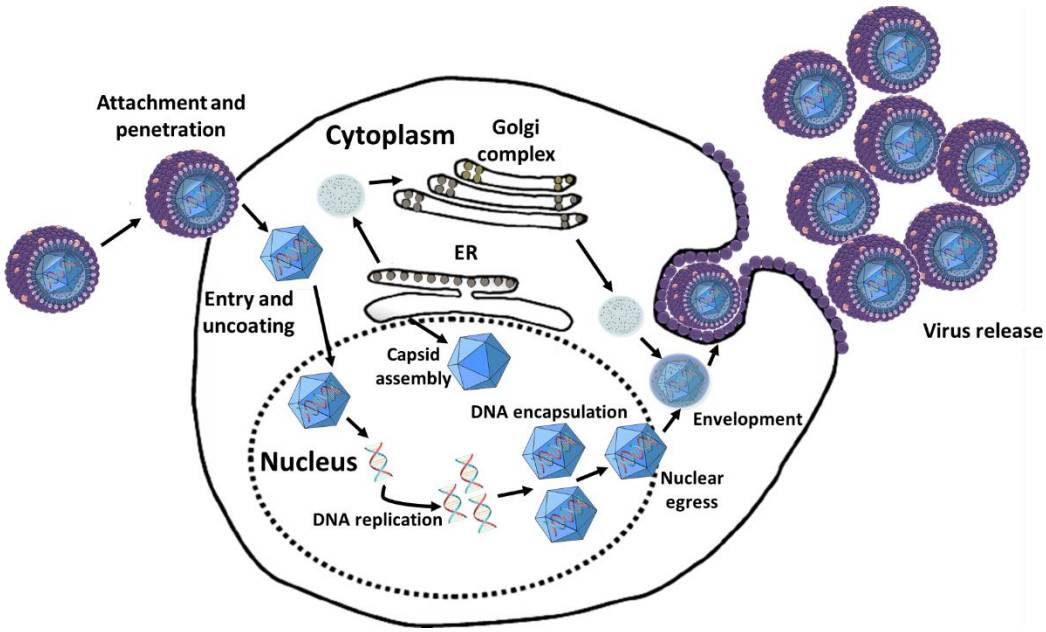

Figure I. 3. HCMV replication summary. Adapted from Ref. [31] .

The receptors for HCMV are widely distributed among host cell types, and contributes to the broad viral tropism observed during natural infections [5]. The first step in this process involves, as mentioned, multiple cell surface components interacting with viral envelope glycoproteins in a process that leads to membrane fusion and delivery of the nucleocapsid to

the cytoplasm of host cells [32]. Binding appears to be part of the essential role of gB, since it exhibit heparin sulfate binding activity, and attachment steps may rely on gH/gL/gO complex[32], has previously stated. This results in fusion between the viral envelope and cell membrane, and release of the nucleocapsid and tegument proteins into the cytoplasm[19]. Once HCMV nucleocapsid is deposited into the cytoplasm, it makes use of normal cytoplasmic transport systems, that control cell shape and vesicular traffic, to control nucleocapsid transit through the cytoplasm. Tubulin-containing microtubules and actin-containing microfilaments facilitate translocation to the nucleus where viral DNA is released. In HCMV, the large tegument protein (LTP, encoded by UL48), as well as a binding protein (LTPbp, encoded by UL47) play essential roles in replication [19,32]. Thereafter, capsid and tegument proteins act in order to allow viral DNA release into the nucleus at nuclear pores. Nevertheless, this process is still under study [32].

During productive infection, HCMV genome is expressed and regulated by a transcriptional cascade of events that lead to the synthesis of three categories of viral proteins called immediate-early (IE), early (E), and late (L) [5,19]. IE gene products play a major role in regulating the expression of early and late viral genes, as well as regulating cellular gene expression, E viral products are generally involved in DNA replication and L genes encode the viral structural proteins, controlling virion maturation [33].

HCMV genes are transcribed in the infected cell nucleus by RNA polymerase II and translation depends entirely on host cell ribosomes [5,19]. HCMV genome replication, inversion, and packaging occurs in the nucleus of the infected cells. Viral DNA synthesis requires the activities of essential and specific viral proteins and the active contribution of several cellular proteins. HCMV owns 6 core replication proteins for viral DNA replication. The single-stranded DNA-binding protein ppUL5, which prevents the reannealing of DNA strands, the helicase complex, which is composed of three subunits (encoded by UL70, UL102, and UL105) and is responsible for unwind the DNA strands, the DNA polymerase encoded by UL54 and the DNA polymerase processivity factor UL44, that prevents UL54 dissociation [5].

In order to maximize viral proteins DNA replication, it is also required proteins encoded by UL84, UL112/113, and UL114. UL84 encodes a protein that interacts with IE2 and stimulates the viral origin (*ori Lyt*)-dependent DNA synthesis. UL112/113 region regulate the establishment of the so-called replication centers corresponding to sub-nuclear sites of HCMV DNA synthesis. Finally, the protein encoded by UL114 expresses a functional uracil DNA glycosylase activity that appears to be required for the process efficiency. During the late stages of viral DNA replication, newly synthesized genomes mature through their inversion, cleavage, and packaging [5].

The formation of HCMV capsids and packaging of viral DNA also occur in the nucleus. Capsid assembly is common to all herpesvirus and requires that capsid shell proteins work in conjunction with the precursor of the assembly protein (pAP). Another protein absolutely essential for encapsidation of viral DNA is the portal protein PORT [32]. During this process, the

nucleocapsids acquire a primary envelopment from nuclear membrane, and after being mature suffer a re-envelopment process in the cytoplasm before leaving the cell via an exocytotic pathway. These nucleocapsid particles accumulate in inclusions that confer the typical ‘‘owl’s eye’’ appearance of the infected cell nucleus [5,32].

As with all herpesviruses, HCMV latency is maintained in everyone who experiences primary infection. The major difference between viral latency and reactivation is characteristically defined by the absence of lytic gene transcription during virus latent carriage[7]. HCMV latency is related with the inactivation of promotors involved on the transcription process. During HCMV latency, the viral major immediate early (MIE) promoter is inactive and the viral genome is associated with enzymes such as histone deacetylase (HDAC) and methyltransferase that are typical of silenced chromatin [37]. In its active form the MIE promotor responds to cellular signal transduction events, the viral genome is associated with histone acetylases that acetylate the histone and open-up the chromatin structure for transcription to occur [34].

HCMV does not have a known latent origin of replication. Genome replication does not appear to be due to any low-level persistence in the cells in which HCMV is carried in vivo [7]. Nevertheless, the balance between natural latency and reactivation is apparently controlled by cell type or cellular differentiation–specific factors that act at both genetic and epigenetic levels to restrict viral regulatory gene expression and replication [19].

I.1.3.3. Pathogenesis and infection Routes

During active HCMV infection and recovery from illness, viral antigen can be detected in many different cells. Usually, HCMV enters in the body via the epithelium of the upper alimentary, respiratory, or genitourinary tracts, once it is transmitted through mucous contact. However, since infection is readily established by transfusion and transplantation, initial infection of epithelial cells does not seem essential[5,6]. After this, HCMV is detected in histopathological sections by visualization cytopathic changes of the infected cells, i.e. the presence of HCMV characteristic owl’s eye inclusion bodies (figure I.4.) [29,35]. These nuclear inclusion bodies were found in lung, pancreas, kidney and in liver. This demonstrate the extensive cytopathogenicity of this infection, and shows that any organ can be infected [36].

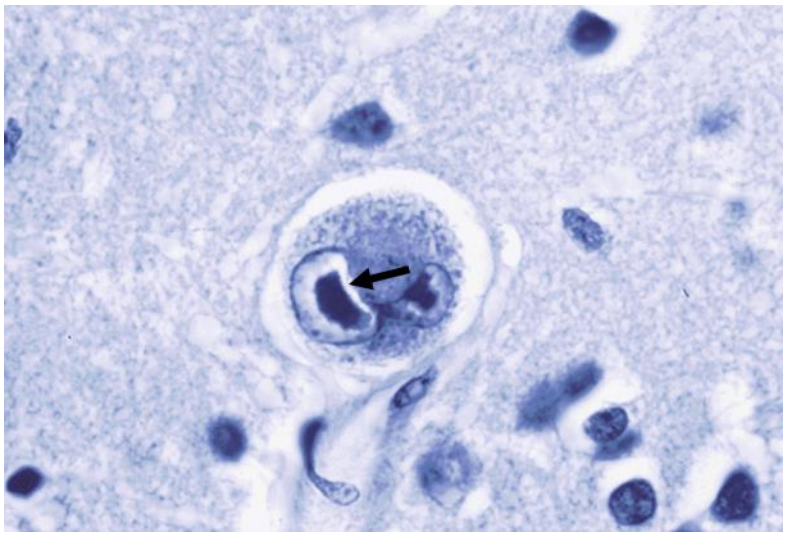

Figure I. 4. Owl's eye inclusion. A single epithelial cell bearing an intranuclear inclusion. Withdrawal from Ref.[29].

HCMV cell tropism present three main group of cells: 1) epithelial cells, endothelial cells and fibroblasts that are the major target for HCMV (highly susceptible to HCMV infection[47]), 2) leukocytes circulating in peripheral blood that are susceptible to the virus (the removal of this cells form the blood of seropositive donors avoid infection by HCMV through blood transfusions [36]) and 3) specialized parenchymal cells such neurons, retina, smooth muscle cells and hepatocytes that can also be infected, leading to significant cytopathogenicity [37].

During the acute infection, HCMV can cause systematically distributed infection, suggesting that HCMV is disseminated hematogenously [36]. The sites where HCMV remains during latent period are still little known [33]. Viral genome have been found in lymphocytes, monocytes and granulocytes, however, none of these cells was permissive to HCMV infection[33,36]. This happens because the virus fails to replicate in poorly differentiated cells. In these cells the virus is maintained in a latent state, with restricted lytic gene expression [33]. It was also observed that epithelial, endothelial and smooth muscle cells during the absence of viral protein expression, were HCMV-DNA positive, suggesting that these cell types might harbor latent virus [38]. Additionally, as previously mentioned, it was demonstrated that leukocytes transmit HCMV infection despite the absence of infectious virus. Peripheral blood monocytes have been shown to harbor latent HCMV naturally. As monocytes are terminally differentiated cells derived myeloid progenitor cells, the up mentioned findings lead to the conclusion that myeloid progenitor cell population might be the latent reservoir of HCMV [38].

Following primary infection, HCMV is excreted in body fluids for months to years [5]. Body fluids include urine, blood, saliva, tears, vaginal and cervical secretion, semen and breastmilk, demonstrating the large number of viral transmission routes and supporting the fact that so diverse cells are infected [5,33]. Indeed, HCMV can be transmitted orally, congenitally, sexually, through exposure to urine, blood transfusion and organ or bone marrow transplant procedures,

being extremely difficult, if not impossible, to distinguish which one was the transmission route [5,6].For HCMV infection there are two main group of risk, persons with immature immune system, like fetuses and newborns, and immunosuppressed individuals, like transplanted ones and persons infected with human immunodeficiency virus (HIV)[7].

Congenital and perinatal HCMV infection results from transmission via placenta, during delivery, or by breastfeeding during maternal viremia [5]. In transplacental transmission, the virus spreads through the fetus by a hematogenous route. Infection during delivery is due to the fetus contact with vaginal and cervical secretions and their possible ingestion. Vaginal and cervical secretions contain high virus titers, as a result of recurrent maternal infection [5]. On the other hand, virus titers are usually low in breast milk, however long-term feeding results in the effective inoculum accumulation and once ingested, the virus infects the mucosa of the oropharynx, esophagus, or the upper airways. For this reason, breast milk is considered one of the main perinatal route of infection [5]. Blood transfusions is an uncommon route for HCMV transmission, but it is known, as already mentioned, that the presence of leukocytes is determinant for infection via blood. Therefore, the current practice of leukodepletion has dramatically decreased the incidence of transfusion-acquired infection[5,36].

Additionally, bone marrow/stem cell transplant and solid organ transplant patients are also at high risk to be infected by HCMV.

I.1.3.4. Host defense

Many pathogens that cause infectious disease in humans multiply in the extracellular spaces of the body, and most intracellular pathogens spread by moving from cell to cell through the extracellular fluids. This extracellular spaces are protected by the humoral immune response, in which antibodies produced by B cells cause the destruction and prevention of intracellular infections [39]. Nevertheless, viruses have no biosynthetic or metabolic system of its own and, in consequence, replicate inside the cells. Once inside the cells, these pathogens are not accessible to antibodies being eliminated by the destruction or modification of the infected cells. This process involves the activation of T-cells or T-lymphocytes. The elimination of infected cells without the destruction of healthy tissue requires the cytotoxic mechanisms of CD8 T cells to be both powerful and accurately targeted [40]. Therefore, during the acute infection by HCMV, immunocompetent individuals develop antibodies and T-cell responses that efficiently control HCMV infection and reduce pathological consequences[9]. However, total HCMV elimination from the body, is never achieved, because the viral genome remains at selected sites in a latent state [5].

Antibodies are essential in defense against infection by pathogens, establishing humoral immunity [41]. The humoral immune response to HCMV is dominated by responses to viral glycoproteins, present in the outer envelope of the virus particle [7]. Several studies demonstrated that a considerable fraction of the virus-neutralizing activity found in human

serum following natural infection is directed against gB and gH. Anti-gH antibodies have a minor neutralizing activity [1,3,5], being estimated that 40 to 70% of the serum neutralizing activity is directed against gB [7,9,30]. The predominance of gB as a target is best explained by its dominant immunogenicity and abundance compared with other components of the envelope.

During primary infection, immunocompetent individuals produce anti-HCMV immunoglobulin (Ig) M class antibodies that persist for 3–4 months, followed a few weeks later by IgG class antibodies that may persist for life [5].

HCMV infection triggers a strong virus-specific cytotoxic T-cell response. CD8+T-cell and natural killer (NK) cells response to the virus are often directed to multiple epitopes [42]. Despite CD8+ T cells and NK cells are both cytotoxic effector cells of the immune system that have the goal to kill infected and transformed cells, the recognition, specificity, sensitivity, and memory mechanisms are considerably different [43].

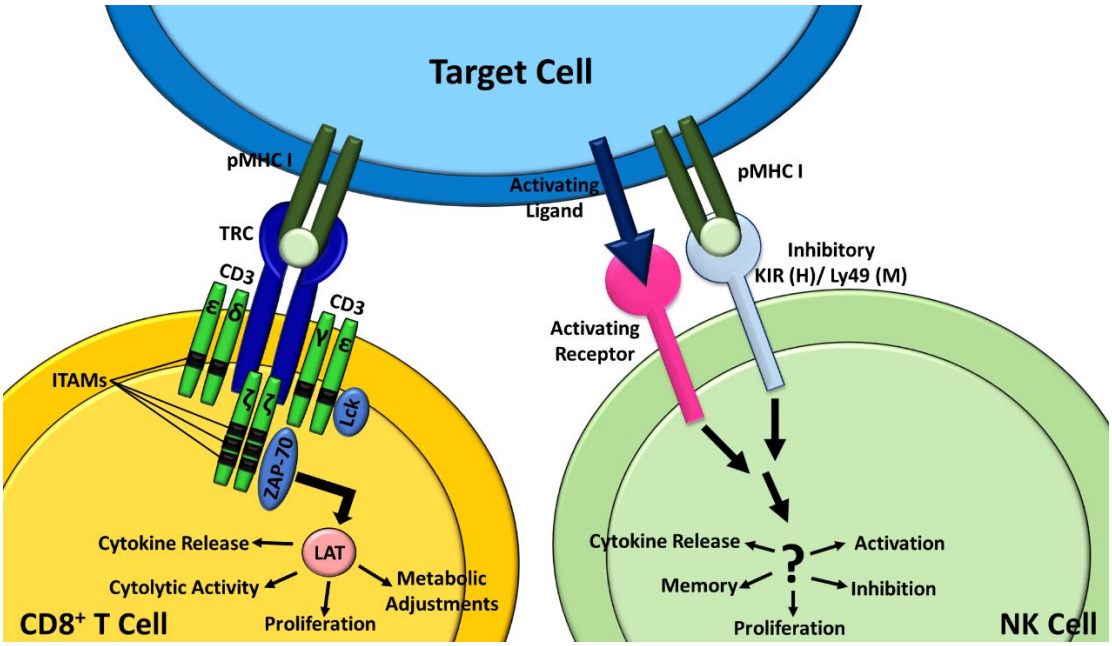

Figure I. 5. a) T cell recognition and signaling. b) NK cell recognition and signaling. NK cell surface activating and inhibitory receptor–ligand interactions mediate the recognition and signaling of a NK cell. The combinatorial threshold that must be reached to activate or inactivate the NK cell is largely unknown. *Adapted from Ref.[43].*

CD8+T cells use their T cell antigen receptors (TCRs) to recognize peptide-major histocompatibility complexes (pMHC) existing on the antigen of the cell surface. Then coreceptor CD8 assists the TCR by binding to the same molecule and this association of both TCR and CD8 with the pMHC triggers the phosphorylation of CD3 (γ, δ, ε and ζ) immunoreceptor tyrosine-based activation motifs (ITAMs) by Lck, a tyrosine kinase associated with the cytoplasmic region of CD8. The phosphorylated CD3 results in the recruitment and activation

of ZAP-70, which in turn phosphorylates LAT. LAT kinase bind with TCR to facilitate signaling during activation and this leads to extensive cellular adjustments, including proliferation, metabolic changes, cytolytic activity, and cytokine release, in other words, controls the activation, differentiation and function of the T cell (figure I.5.) [43].

NK cell recognition is much less understood. In contrast to T cells, there is no single dominant receptor to mediate NK cell recognition. Rather, NK cells express an innate activating and inhibitory receptors to sense their environment and respond to alterations caused by infections, stress and transformation [43]. What is known is that NK cells are cytotoxic lymphocytes that are important because many DNA viruses encode molecules that are able to evade from pMHC I processing, avoiding its recognition by CD8+T cells, and they fulfill the gap in immunosurveillance by CD8+T cells thanks to the called "missing self" mechanism [44]. This mechanism postulates that the absence or incomplete expression of host major histocompatibility complex (MHC) class I molecules in a normal target cell would be sufficient to render it susceptible to NK cell (figure I.5.) [43,45].

In this way, HCMV-specific cytotoxic T lymphocytes (CTL) response is required for recovery from HCMV infection. Suppression of CTLs caused reactivation and dissemination of natural infection, being CD8+T-cells and NK cells of primary importance in the prevention of recurrence. And since HCMV infections are most severe in patients with dramatically impaired cell-mediated immunity, such as one marrow transplantation (BMT) recipients and those with HIV, it is evident that this immune response provides the great protection [5].

I.1.3.5. Seroprevalence

HCMV seroprevalence generally increased with age [15]. The majority of primary HCMV infections occurs early in infancy. After the second year of life, non-maternal sources of infection may enhance viral transmission. Close contact among a high number of children at kindergartens, may be a possible mode of viral spread during childhood [41]. During adolescence an increase in HCMV seropositivity is also observed, suggesting a sexual component for virus transmission, but individuals between 30 and 35 years of age represent the group with the highest risk of a HCMV seroconversion [41,42]. Also, as previously mentioned, HCMV infection is relatively common among women of reproductive age, with seroprevalence ranging from 45 to 100% [6]. In the case of global congenital HCMV infection, seroprevalence is 0.3–0.7% of all live births [46].

The risk of congenital HCMV transmission is highest in a pregnant woman who acquires primary HCMV infection during pregnancy. Approximately 30–39% of primary maternal HCMV infections results in transmission of virus to the fetus, and up to 13% of these infections will result in symptomatic congenital disease in the newborn [46]. However, fetus transmission and consequent negative effects vary with gestational age at infection, since the risk of symptomatic congenital infection was mainly connected with maternal first and second

trimester infection [46,47]. Recurrent HCMV infections in pregnant women (either through reactivation of latent virus or re-infection with a new strain of HCMV) can also lead to congenital HCMV transmission and fetal infection, however, with a lower transmission rate (approximately 1.4%) [46]. This suggests that populations with higher seroprevalence rates may have a lower risk of primary maternal HCMV infection and therefore lower rates of symptomatic congenital HCMV [46].

The worldwide geographic patterns of HCMV prevalence also presents some variety. HCMV seroprevalence tended to be elevated in South America, Africa and Asia, in parts of Europe (e.g. Italy and Sweden) and the Middle East (e.g. Turkey and Israel), being lowest in Western Europe and in the United States (figure I.6.) [6]. Despite the fact that some developed countries present high rates of HCMV seroprevalence, generally, it can be said that HCMV prevalence is higher in developing countries and socio-economic factors such as poor nutrition, inadequate health and hygiene care may contribute to the higher transmission rates[46,48]

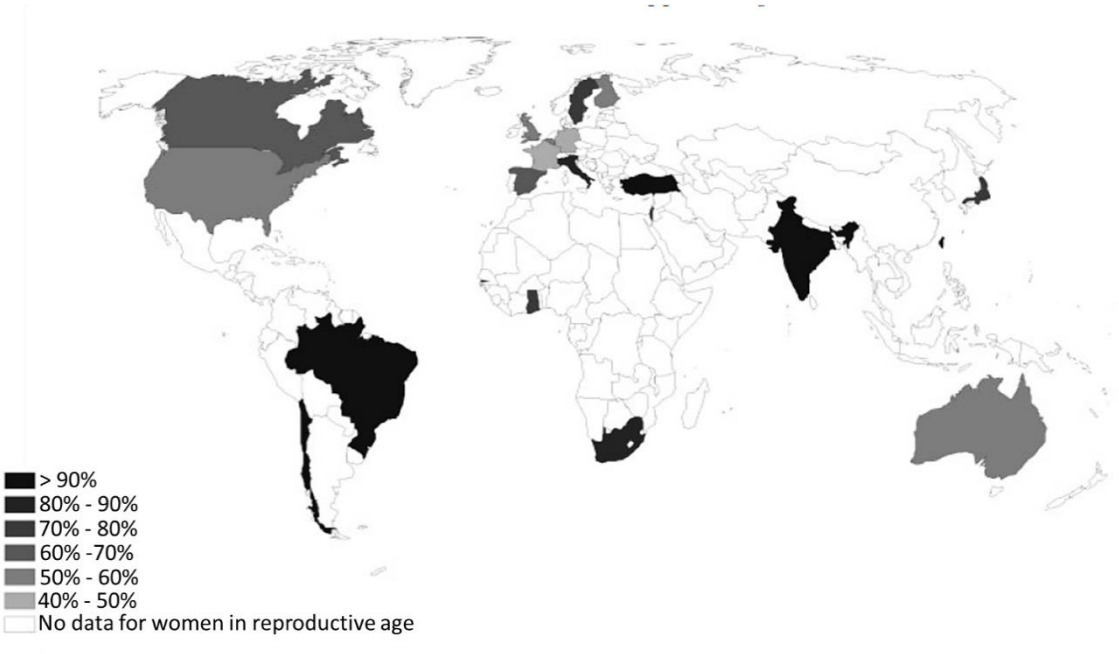

Figure I. 6. Worldwide HCMV seroprevalence among women of reproductive age. Reproductive age was generally defined as between 12 and 49 years of age. Adapted from Ref. [6].

I.1.3.6. Prevention of HCMV infection

In order to prevent HCMV infection in immunosuppressed individuals and pregnant women, who are susceptible to HCMV infection, individuals should be advised of the importance of careful hand washing and cleaning of environmental surfaces. Also, if blood transfusions are required, they should always receive HCMV-negative blood, and this same type of blood should

also be used for any intrauterine transfusion. In addition, because of the possibility of HCMV transmission through sexual intercourse, safe sexual practices should be encouraged [49].

Cytomegalovirus remains the major infectious cause of congenital abnormalities in the developing fetus and newborn, being a public health problem [1,39]. In this way, a first logical step for public health action towards awareness, prevention and treatment of HCMV infection is to screen all pregnant women and newborns. Routine prenatal screening of all pregnant women for the presence of HCMV IgG antibody would increase HCMV awareness, because the mere action of conducting HCMV antibody testing requires discussing the results with the mother during her prenatal visits and provides the opportunity to introduce preventive behaviors for the vulnerable HCMV seronegative mothers. In such wise, pregnant women and newborn screening programs for congenital HCMV infection and disease should be established [50]. Nevertheless, nowadays all of the usually used methods for HCMV detection require a long period of time to perform or are costly, being problematic to be established in screening programs [50,51].

Another way to solve the problem of HCMV congenital infection is by means of active immune prophylaxis, i.e., vaccination [1]. Thus, the ultimate goal of a HCMV prevention program should be the development of a vaccine to be administered to seronegative women of childbearing age to prevent the occurrence of primary HCMV infection during pregnancy [4], this will also apply to other individuals. Attempts to develop an HCMV vaccine have been directed at five major strategic approaches: 1) live attenuated vaccines; 2) subunit vaccines; 3) peptide vaccines; and 4) DNA vaccines [1].

Live attenuated vaccines express a full or nearly full complement of viral antigens, as consequence they can induce both humoral and cellular immune responses, that closely mimic those induced by natural infection. However, safety is a significant concern for a live HCMV vaccine, especially if inadvertently administered during pregnancy [52]. Several problems may be faced, vaccine virus strain may persist in the body as a latent virus and periodically reactivate, animal models for HCMV are not available and HCMV may be oncogenic *in vivo*, as suggested by its ability to transform both human and embryonic hamster cells *in vitro* [1]. The Towne vaccine is a live attenuated vaccine that is effective in preventing severe HCMV-associated disease in renal transplant recipients. However, when used at low doses, this vaccine failed to protect seronegative women of childbearing age against wild-type HCMV, probably due to lower titers of vaccine-induced neutralizing antibodies [53].

In an attempt to improve the immunogenicity of the Towne vaccine, the genomes of Towne and the non-attenuated Toledo strain were recombined so that, regions from the low-passage Toledo strain of HCMV were substituted for the corresponding regions of the Towne genome to produce 4 different “Towne-Toledo chimeras”. Towne/Toledo chimera vaccines were well tolerated in HCMV-seronegative subjects and were greatly attenuated relative to the Toledo strain or to wild-type HCMV [54].

Subunit vaccines provide potent and focused immune responses to select viral immunogens. The simplest subunit vaccines combine subunit immunogens (e.g., recombinant proteins) with

an adjuvant [52]. In this respect, major antigenic sites of the immune response to HCMV which are potential components of a subunit vaccine are viral glycoproteins gB (UL55) and gH (UL75), the principal targets of the neutralizing antibody response, and viral phosphoproteins pp65 (UL83) and pp150 (UL32), which are the dominant targets of the cytotoxic T-lymphocyte (CTL) immune response[1]. In the light of this, Pass et al. [55] conducted a Phase II placebo-controlled, randomized, double blind trial (Level I evidence) of a new HCMV vaccine. This vaccine was prepared by recombinant technology and contained envelope glycoprotein B along with the MF 59 adjuvant. 234 subjects received the HCMV vaccine and 230 subjects received placebo. After a minimum of 1 year of follow-up, there were 49 confirmed infections, 18 in the vaccine group and 31 in the placebo group. From these, one congenital infection among infants of the subjects occurred in the vaccine group, and three infections occurred in the placebo group. These results showed that this vaccine has potential to decrease incident cases of maternal and congenital HCMV infection. Although the sample size for this study was considered not large enough to test a hypothesis concerning congenital infection, newborns of subjects were tested for CMV infection. Peptide vaccines are based on the use of peptide fragments of immunogenic viral proteins, referred to as minimal cytotoxic epitopes. However, peptide vaccines have a disadvantage of limited efficacy due to their limited specificity. Additionally, this minimal cytotoxic epitopes had to be suspended in a strong adjuvant to be able to elicit an efficient immune response [1]. Diamond *et al.* developed a peptide-based vaccine against HMCV. They mapped the epitope from HCMV pp65 and verified in *in vitro* studies that pp65495-503 peptide has stimulated significant recognition of HCMV infected cells. Also studies using a transgenic mice showed immunization with this peptide [56].

DNA vaccines are based on the expression of heterologous genes *in vivo*, and these genes are carried by plasmid vectors. Results of DNA vaccination are determined by both the efficiency of delivery and the level of expression of the heterologous gene[1]. Transvax™ vaccine was developed by Vical. It is comprised of plasmid DNA encoding pp65 and gB. This vaccine was formulated with CRL1005 poloxamer and benzalkonium chloride, which is a delivery system that enhances gene expression *in vivo* and increases immune responses compared with DNA vaccines formulated in phosphate buffered saline alone. The beneficial effects of TransVax compared to placebo, combined with an acceptable safety profile, suggested that this vaccine might fill the unmet need for an effective cytomegalovirus vaccine [4].

I.1.3.7. Clinical features and management of patients

Infection with HCMV in immunocompetent individuals is usually not diagnosed because it is either asymptomatic or is associated with a mild “flulike” illness [3]. However in some cases

symptoms like persistent fever, myalgia, head ache, cervical lymphadenopathy, splenomegaly and rash may appear and persist for weeks [5].

In the case of immunosuppressed individuals, the risk of HCMV infection severity is relate to the serologic status of the transplant donor and recipient, the likelihood of receiving HCMV seropositive blood products, and the degree of immunosuppression that the transplant patient experience [57]. In transplant recipients, clinical signs of HCMV infection is now less frequent as a result of better prophylaxis. However, when its transmission occurs through this via, the disease is initially localized in the transplanted organ, i.e., hepatitis occurs generally in liver transplant recipients, pancreatitis in pancreas transplant recipients, but then spreads throughout the gastrointestinal tract, lungs, and central nervous system (CNS)[5]. When compared with solid organ transplant recipients, bone marrow transplantation (BMT) recipients are the most difficult group to treat [5]. The high rate of severe HCMV disease after BMT is related to the immunodeficiency post-transplant, especially when it results in interstitial pneumonia [57,58]. Furthermore, post-transplantation HCMV infection appears to increase the risk of fungal superinfection, probably as a result of the leukopenia that complicates HCMV infection [57].

HCMV is also a common cause of serious opportunistic viral disease in patients with human immunodeficiency virus (HIV) infection [59]. HCMV disease in HIV patients most often affects the digestive system, causing esophagitis, enteritis, gastritis and colitis [5,60]. Other manifestations include renitis, hemolytic anemia, myocarditis, peripheral neuropathy, polyradiculoneuritis, pneumonitis and hepatitis [60].

Finally, HCMV infection is the most frequent congenital infection worldwide [51]. Congenital HCMV infection may cause complications during pregnancy such as fetus growth restriction and birth defects [61]. Congenital HCMV infection results from transplacental transmission of the virus during maternal viremia and after transplacental transmission, the virus spreads through the fetus by a hematogenous route[51]. Due to HCMV latency, transplacental fetus infection can occur both in women infected for the first time during pregnancy (primary infection) and those infected long before conception (recurrent infection)[1,5,51].

Irrespective of the type of maternal infection, the majority of congenital HCMV infections are subclinical. Only 10% of the estimated infants with congenital HCMV infection exhibit clinically apparent or symptomatic infection at birth, and the vast majority of infected newborns have no detectable clinical abnormalities (only 15% of the infected may develop hearing defects or impaired intellectual performance) [5,62]. Reactivation is a more frequent cause of congenital infection than primary maternal infection, however, those resulting from a recurrence of HCMV during pregnancy are less likely to be clinically apparent than those resulting from a primary infection [5,63].

It is commonly recognized that primary HCMV infections are transmitted more frequently to the fetus and are more likely to cause fetal damage than recurrent infections[1]. Most children with symptomatic congenital HCMV infection (approximately 80%) develop one or more sequelae including sensorineural hearing loss, mental retardation, motor deficits, seizures, and chorioretinitis [62]. Transplacental HCMV fetus transmission, resulting from primary

infection, ranges from 20% to 40% of the cases, whereas the transmission rate after recurrent infection is about 1 to 3% [5,51].

In addition, it seems that primary infection occurring at an earlier gestational age is related to a worse outcome [1]. Transmission rates in the first trimester were from approximately 35% to 40%, in the second trimester were about 45% and in third trimester were about 65% to 75% [47]. In this way there is an increased risk for *in-utero* transmission of HCMV in the third trimester. Nevertheless, the risk of symptomatic congenital infection was mainly connected with maternal first and second trimester infection [47]. In this way, the severity of congenital disease is inversely related to gestational age and is much greater when infections occur in the first and second trimester [64,65]. The possible reason for this is that sequelae of fetal infection are worse during organogenesis and during neuronal growth (that are completed at about 26 weeks of gestation), so as the duration of pregnancy increases, fetal infections are less likely to cause congenital malformations [64].

It should also be noted that despite the fact that symptomatic congenital infection and permanent neurologic deficits being rare in the infants of women with preconceptionally immunity, it is also important to define the factors that are associated with intrauterine transmission of HCMV in women with preconceptional immunity [66]. Although recurrent infection is not as likely to induce permanent sequelae in the fetus as primary infection, it must be emphasized that recurrent infections may consist of either reactivation of the same virus strain or reinfection by a new virus strain. When recurrent infection occurs by a new virus strain, the incidence of symptomatic congenital HCMV infections in mothers with preexisting immunity has been shown to be similar with primary infection[1,66]. On the other hand, congenital infections following reactivated maternal infection are mostly asymptomatic[1].

Since HCMV infection is the most frequent cause of embryonic and fetal pathology, the management of pregnant women with HCMV will be highlighted. Once an acute or recent primary HCMV infection is diagnosed with certainty or high probability, the woman is given complete information about the risks of transmission, possible clinical outcome for the child, therapeutic possibilities in the case of symptomatic disease at birth, as well as prenatal diagnosis (if gestation time allows this option) [1]. Possibilities and limitations of prenatal diagnosis are discussed, considering the timing of maternal infection, certainty of diagnosis, including the event of a possible false-negative result, and time of gestation. The woman is also informed about the possibility of terminating the pregnancy [1].

During prenatal testing, the women undergoes biochemical/hematological, virological, and ultrasound testing. Clinical samples currently used for prenatal diagnosis are fetal blood drawn by cordocentesis and amniotic fluid obtained by amniocentesis [1]. Fetal imaging by ultrasound can identify structural and/or growth abnormalities associated with intrauterine HCMV infection [60]. Ultrasound allows to detect cerebral abnormalities such as cerebral ventriculomegaly, brain calcifications, microcephaly and occipital horn anomalies, and noncerebral abnormalities such as echogenic bowel, intrauterine growth restriction,

hepatomegaly, ascites and cardiomegaly. However, other features of congenital HCMV disease such as chorioretinitis, petechiae and neurodevelopmental defects are not detectable by fetal imaging, therefore, the absence of fetal abnormalities does not exclude fetal damage. Additionally, HCMV is also associated with fetal death where ultrasound features of infection may be less obvious [46]. Prenatal diagnosis allows the understanding of the natural history of congenital HCMV infection in order to better prepare the family to face the health problems of the infant or young child. Most of the non-neurological disease in newborns can be treated. In contrast, the neurological damage is permanent and accounts for the long-term morbidity [5]. Prenatal diagnosis also represents the step preceding the potential introduction of antiviral therapy. In principle, two levels of treatment could be considered, prenatal (during fetal life) and postnatal (based on severity of clinical symptoms) and the antiviral drugs available are ganciclovir, foscarnet and cidofovir [1]. Ganciclovir (and its orally available formulation, valganciclovir) is a guanosine analog that, after phosphorylation by the HCMV UL97 kinase, acts as a chain terminator during viral DNA replication. The nucleoside monophosphate analog cidofovir and the pyrophosphate analog foscarnet also inhibit viral DNA polymerase activity, but neither requires prior activation by any other viral protein [4]. Ganciclovir products have been tested most widely in randomized controlled trials in both transplant and HIV-infected subjects. In addition to intravenous and oral formulations, ganciclovir can be given locally to the eye in patients with sight-threatening retinitis. Systemic ganciclovir's principal toxicity is neutropenia. Although foscarnet is as effective as ganciclovir, its main side effects are renal toxicity and electrolyte imbalance [4]. Cidofovir has been shown to be effective in the treatment of HCMV retinitis. Nevertheless, there is the possibility of developing drug resistance with all available drugs [4].

Considering now the transplanted patients and other immunosuppressed individuals, the availability of effective prophylactic and preemptive antiviral therapy has made HCMV a rare cause of mortality [7].

I.1.3.8. Diagnosis

The diagnosis of HCMV disease remains controversial because of the difficulty of separating patients who are asymptomatic but shedding HCMV in body fluids, like urine and saliva, from patients who have symptomatic disease, such as pneumonia or retinitis, for which therapy might be warranted [3]. As mentioned, this virus can be detected in blood, urine, saliva, stool, tears, breast milk, cervical and vaginal secretions, and semen [3].

The laboratory diagnostic methods for evaluating HCMV infection can be divided into serologic and virologic techniques (table 1.2.). Serologic methods are used mostly to assess the antibody status of the donor or recipient in transplant situations and to predict the risk that immunocompromised patients will develop symptomatic disease under various conditions[3,67].

During the acute infection by HCMV viral nucleic acid translation and transcription occur intracellularly resulting in viral proteins generation. Both IgM and IgG antibodies are produced against these protein antigens[10]. As consequence, humoral immunity is important to detect an infection by HCMV, also allowing the identification of a past and a recent HCMV infection, through serological determination. Serological determination rests on detection of virus-specific IgM or IgG antibodies [5,68]. Elevated titer of IgM antibodies appear during the active infection and are highest during viremia, while IgG is valuable for establishing past infections and its presence indicates former infection from 2 weeks to year's duration [60,68].

A specific IgM response was usually associated to a serologic evidence of a recent primary infection. But the reality is that IgM is associated with both primary and reactivated infection[68]. Additionally, the discovery of HCMV–IgM antibodies in a first serum does not allow the diagnosis of an actual infection, since, HCMV–IgM antibodies can persist for months after primary infection [65]. Apart from this, detection of IgM antibodies is not reliable because false positives may be induced by rheumatoid factor, antinuclear antibodies, and other cross-reactive factors not yet identified. Nevertheless, increases in levels of IgM antibody provide a marker for virus replication in patients and IgM synthesis deficiencies may be associated with severe HCMV disease in some immunocompromised patients [68,69]. In this way, the occurrence of a primary infection is conventionally deduced from seroconversion from IgG antibody negative to IgG antibody positive in the interval between two serological assays [5]. The interpretation of a positive IgM assay is assisted by determination of the avidity of HCMV-specific IgG. Low-avidity IgG is produced early in infection and high- avidity IgG is a marker of past or recurrent infection [18,69,70].

Owing to the fact that immunoglobulin A (IgA) antibodies presented higher titers during recent infection, persisting longer than IgM, but with a more rapid declination in titers when compared to declination of IgG titers, IgA was considered appropriate to be used in clinical situation as suggestive of recent HCMV infection. However, their response duration fluctuate considerably, making difficult to interpret the role of IgA during HCMV infection[71,72]. In this way serologic diagnosis of HCMV infection is most often based on titers of HCMV- IgG, or HCMV-IgM detection [72].

Many different assays are available for HCMV antibodies, being enzyme-linked immunosorbent assay (ELISA) the most widely used [1]. ELISAs have been used in both the indirect ELISA and the capture ELISA format with either labeled antigen or antibody. The overall specificity of this method was 98.9% and the sensitivity 100% by detecting antibodies in the blood. However, there is the possibility of false positive results, caused by cross-reactions with some virus of *Herpesviridae* family, and rheumatoid factors [1].

On the other hand, virologic diagnosis of HCMV disease is usually based on the isolation of virus by culture methods [3]. This approach utilizes clinical samples which are inoculated onto a monolayer of human fibroblast cells and incubated for a period of time ranging from 2 to 21 days [3,60]. HCMV infected cells can be identified by owing ''owl's eye'' inclusions [5] (figure I.4).

The cytopathic effect observed in the cells is directly related to a virus's titer. However, this method is slow and requires 2–3 weeks until a result can be reported as negative [60].

Shell vial assay is a modified viral culture by using a centrifugation technique designed to decrease the time needed for virus detection. Centrifugation of the samples onto the cell monolayer greatly assists adsorption of virus, increasing infectivity of the viral inoculum. Viral growth is measured by antigens detection through the use of monoclonal antibodies. Virus detection is made by indirect immuno-fluorescence after 16 hours of incubation. This method was adapted to be performed in 96-well microtiter plates allowing for the screening of larger numbers of samples [60].

HCMV-infected cells can also be identified more efficiently, by immunohistochemistry, polymerase chain reaction (PCR) and nucleic acid hybridization [5].

Immunohistochemistry is performed primarily on tissue or body fluid samples. Slides are made from frozen sections of biopsy tissue samples (e.g. liver, lung) or by centrifuging cells onto a slide. Then monoclonal or polyclonal antibodies against early HCMV antigens are applied and visualized by fluorescently labeled antibodies or enzyme labeled secondary antibodies which are visualized by the change of color of the substrate. The stained slides are then examined by fluorescent or light microscopy. This technique is more sensitive and very specific compared to plain histological microscopy, but it is very labor intensive and requires experienced personnel to read the slides. False negative results can also occur [60].

PCR is a rapid and sensitive method for HCMV detection. The technique is based on amplification of selected segments of the HCMV genome and its hybridization [5]. The techniques usually target antigen genes in their well conserved regions [60]. DNA samples can be extracted from whole blood, leucocytes, plasma, any other tissue or body fluid[60]. Additionally, PCR for HCMV DNA can be either qualitative or quantitative [60].

Reverse transcriptase (RT-PCR) can be used to detect viral mRNA transcripts in leukocytes independent of the presence of DNA. The absence of circulating mRNA is associated with a lack of HCMV-associated symptoms [60]. With RT-PCR, false-positive can occur, most likely due to the difficulty in differentiating between RNA and DNA derived PCR products in the case of unspliced transcripts [1]. This methods are generally more expensive [60].

Among the nucleic acid hybridization techniques amplifying the signal generated rather than the use of viral DNA itself, two have become commercially available for quantification of HCMV DNA: the digene hybrid capture system HCMV DNA assay and the branched DNA assay. The hybrid capture system is based on the formation of a DNA-RNA hybrid which is captured by a monoclonal antibody specific for the hybrid and is then reacted with the same monoclonal antibody labeled with alkaline phosphatase. The hybrid is finally detected with a chemiluminescent substrate, whose emission is proportional to the amount of target DNA present in the sample. This hybrid capture system assay has been reported to have increased sensitivity [1,73]. The branched DNA assay is based on the use of branched DNA amplifiers containing multiple binding sites for an enzyme-labeled probe. The target DNA sequence binds

to the branched DNA molecule, and the complex is revealed by a chemiluminescent substrate whose light emission is directly proportional to the target DNA present in the sample [1,74].

Finally, another technique commonly used for HCMV virus quantification in blood samples is the antigenemia assay[60]. Antigenemia is measured by the quantitation of leukocyte positive nuclei, in an immunofluorescence assay, for the HCMV lower matrix phosphoprotein pp65 in a cytospin preparation of 2×10^5 peripheral blood leukocytes (PBL). [5]. The viral pp65 antigen is a structural late protein expressed in blood leukocytes during the early phase of the HCMV replication cycle [60]. High antigenemia levels are often associated with HCMV disease and the assay is widely used for monitoring of HCMV infections and antiviral treatment. The major advantage of the antigenemia assay is rapidity in providing results in a few hours, while major disadvantages are the labor procedure, not amenable to automation, limited number of samples processed per test run and the subjective component in slide reading [1].

Table I. 3. Summary of diagnostic tests for identification of HCMV.

DIAGNOSTIC TEST	INFECTION INDICATOR	ADVANTAGES	DISADVANTEGOUS
IGM/IGG SEROLOGY	IgM positivity IgG Seroconversion Low IgG avidity	Indication of primary maternal infection	Possibility of false-positives
FIBROBLAST CULTURE	Presence of inclusion bodies on the cells	The cytopathic effect is directly related to virus titer	Requires 2–3 weeks for a diagnostic
SHELL VIAL	Presence of antigens on the sample	Fast diagnostic Allow the screening of a large number of samples	Possibility of false-positives. Reading stained preparations is time-consuming and labor-intensive
IMMUNOHISTOCHEMISTRY ASSAYS	Presence of antigens on the sample	It is possible to use fresh or frozen tissue sample Fast diagnostic	Reading stained preparations is time-consuming and labor-intensive. Possibility of false negative
PCR	HCMV DNA positive	Excellent sensitivity and specificity	Technical expertise required Expensive method and instrumentation
RT-PCR	HCMV mRNA positive	Easier automation, and processing of large numbers of samples.	Technical expertise required Expensive method and instrumentation Poor sensitivity, reproducibility and specificity
NUCLEIC ACID HIBRIDIZATION	HCMV DNA positive	Excellent sensitivity and specificity	Technical expertise required Expensive method and instrumentation
ANTIGENEMIA ASSAYS	Presence of pp65 antigen on the sample	Fast diagnostic	Reading stained preparations is time-consuming and labor-intensive Limited number of samples processed per test
ULTRASOUND (PREGNANT WOMEN)	Identification of fetal abnormalities, when HCMV infection is confirmed	Non-invasive Allows to see symptomatic fetal infection	Not specific for HCMV

I.2. Immunoassays

I.2.1. Immunoassays overview

The basis of all immunoassays is the ability of antibodies to form complexes with the corresponding antigens. This property of highly specific molecular recognition of antigens by antibodies leads to high selectivity of these assays [75]. In these techniques, one of the immunoagents (antigen or antibody) is usually immobilized on a solid support and the immunoreaction results in the formation of antigen-antibody complex [75]. Afterwards, quantification of the bound immunoagent of interest is conducted by using labels, with specific properties suitable for detection, that are covalently bound to the antigens or antibodies [75].

During the last decades there has been an increase in the variety of immunodiagnostic tests [76,77]. There are three widely accepted assays that employ labelled antibodies and antigens. These assays are immunofluorescence, in which a fluorescent dye is conjugated to the antibody; radio-immunoassay, in which isotopes are attached to antibodies or antigens and enzyme immunoassays, that employs antibodies or antigens conjugated to enzymes in such a way that the immunological and enzymatic activity of each moiety is maintained [77,78].

In practice immunofluorescence is not easy to quantify for antibody assays, since it can be difficult the visualization of the lesions in tissue and mostly immunofluorescence assays have limited sensitivity and specificity [79,80]. Radioimmunoassay is highly sensitive and allows precise quantification. However, the isotope labels may decay rapidly, so that the conjugates have a short shelf-life. Additionally, complex equipment is necessary for their assessment and, because of the medical hazards, they must be handled only by highly trained personnel. Finally, enzyme immunoassays give objective results that can be analyzed by a simple spectrophotometer, requiring just few hours of analysis time and the required reagents are stable, presenting long shelf-lives, and are not health hazards. These advantages make this technique the gold standard for immunoassays [76-78].

I.2.2. Antigens and antibodies

The specificity of the molecular recognition of antigens to form stable complexes is the basis of analytical immunoassays [81]. The binding pattern in the case of antibody-antigen complex is similar to that found with other protein-protein interactions [82].

I.2.2.1. Antigen

Antigens (Ags) may be defined as structures which generate an immune response. They can be recognized, as previously mentioned, by three elements of the immune system: antibodies, T cells receptors and MHC molecules.

While T cells and MHC molecules bind to short peptides that are essentially linear in structure and that can derived from any part of the molecule, antibody-antigen interaction is shape dependent[83]. Antibodies bind antigens over large sterically and electrostatically complementary surfaces. Van der Waals forces, hydrogen bonds, and occasionally ion pairs provide stability to antibody-antigen complexes. In addition, water molecules contribute hydrogen bonds linking antigen (Ag) and antibody, and increase the complementarity of antigen-antibody interfaces [84]. The part of the antigen which the antibody interacts is termed the epitope. This region determines which antibody will bind so it can also be referred as an antigenic determinant. The overall strength of binding between an antibody and an antigen is called avidity [83].

I.2.2.2. Antibody

Antibodies (Abs) which belong to a group of large polypeptides termed immunoglobulins that are generated by B lymphocytes and plasma cells. The name immunoglobulin derived from the fact that they are globular proteins (globulins) that have an immune function. [83]. Abs have two distinct functions: to bind specifically to their target Ags and to elicit an immune response against the bound Ag by recruiting other cells and molecules [85].

I.2.2.2.1. Antibody structure

The typical immunoglobulin molecule has a molecular mass of 150-200 kDa. Its general structure shows a Y-shape consisting of three equal-size portions, loosely connected by a flexible hinge. They are also constituted by two heavy (50-80 kDa) and two light (approximately 23 kDa) polypeptide chains. For the assembly of a full antibody molecule, both heavy chains are linked together by two disulphide bond and each heavy chain has a light chain also attached by a disulphide bond (figure I.7.) [83].

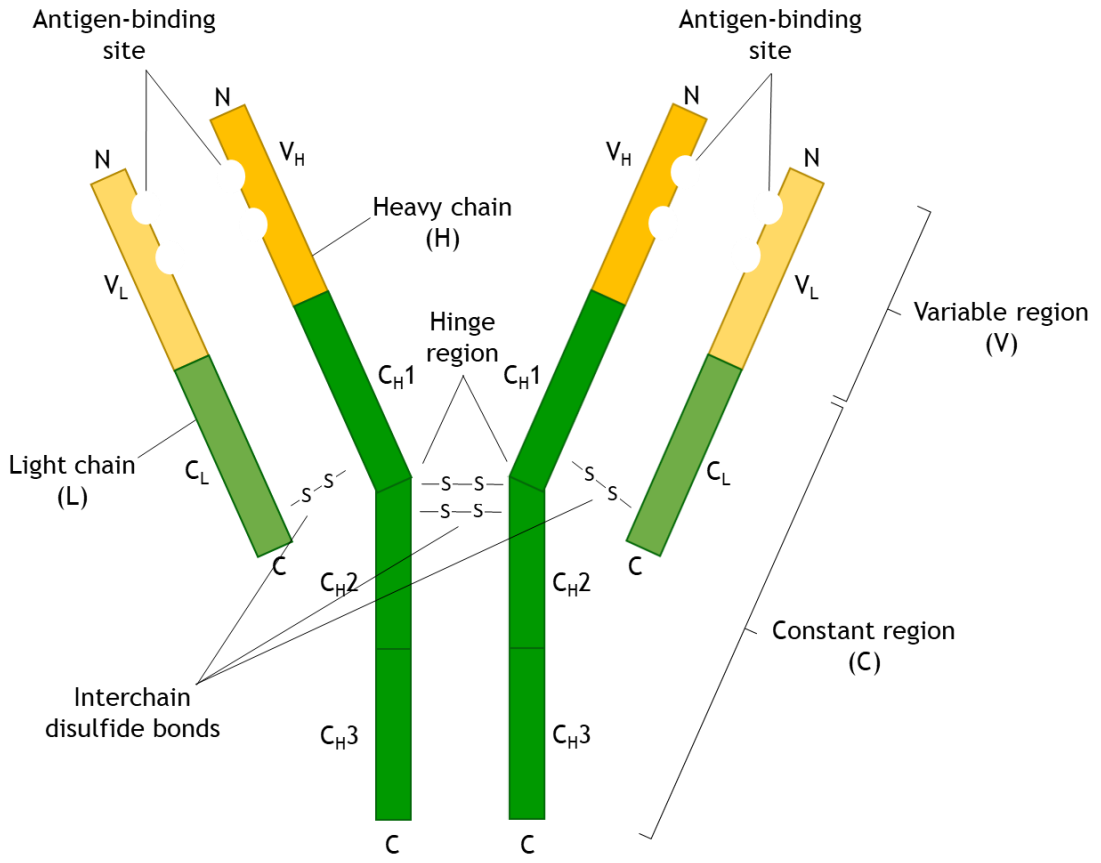

Figure I. 7. Antibody structure. Adapted from Ref. [86].

The heavy and light chains have a relatively stable and conserved segment, which varies little between different antibodies. These relatively constant regions are called C regions, and they hold the effector functions of the molecule and determine the isotype of the antibody [83,85]. On the other hand, there also exists regions in which the amino acid sequence varies enormously from antibody to antibody. These regions are called V regions and bear three hypervariable regions, known as complementarity-determining regions (CDRs), which are responsible for the specific antibody–antigen interaction and where we can find the antigen binding sites (ABS) [85]. This variability is essential for generating the potential to bind to more than 10^{11} different antigen structures [83]

The both arms of the Y-shape structure are knowns as Fab. The Fab is composed of two variable domains (V_H in the heavy chain and V_L in the light chain) and two constant domains (C_H1 and C_L) [85]. The two additional domains of the heavy chain, C_H2, and C_H3, compose the Fc region of the Ab molecule [85]. There is also a hinge region in the middle of the molecule that allows some freedom to the both arms that bear the ABS (figure I.7.). This flexibility allows to maximize the chances of binding between the Ab and the antigenic epitopes [83].

I.2.2.2.2. Classes of antibodies

Different classes of immunoglobulins have been identified on the basis of physicochemical properties and the distinct antigenic specificities of their heavy chains. Each class may contain several related types (or subclasses) (table I.4.) [87]. These classes are defined by the type of the heavy chain. Currently there are five types of mammalian Ig heavy chain (γ, α, μ, δ and ε) and they differ in size and composition. α and γ contain approximately 450 amino acids, while μ and ε have approximately 550 amino acids [82,83,88].

IgG has a γ chain, is the most abundant immunoglobulin and is also the most used antibody in immunosensig assays. IgG occurs as a monomer and can be subdivided into four subclasses [83,89]. IgG subclasses, named in order of decreasing abundance, are IgG1, IgG2, IgG3, and IgG4 [89] (Fig. I.8.). The variability between the four IgG subclasses is manly located in the hinge regions and functional domains [83]. IgG1 responses to soluble protein antigens and membrane proteins primarily, but are accompanied with lower levels of the other subclasses, mostly IgG3 IgG4 [89]. IgG2 response is important in combating capsulated bacteria [83,89]. Ig3 is a potent pro-inflammatory antibody, its shorter half-life may function to limit the potential of excessive inflammatory response [89]. Ig4 antibodies are often formed following repeated or long-term exposure to antigen in a non-infectious situation [89].

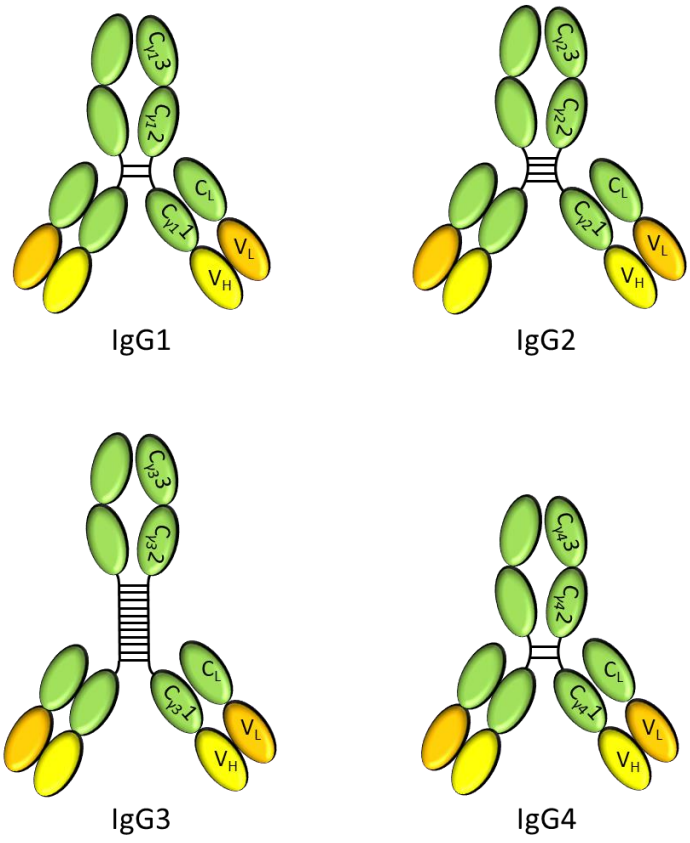

Figure I. 8. The four subclasses of human IgG. Adapted from Ref. [83] .

IgA has a α chain and is the second most abundant immunoglobulin molecule. It can occur not only as a monomer but also as a dimer, in which two IgA molecules are joined by a short peptide (J chain). This immunoglobulin can be secreted onto the external surfaces (secretory IgA), being important in the defense of the mucosal surfaces of the host. Although IgA is detected abundantly in serum (usually in the monomeric form, IgA1), it can also be found (in dimeric form, IgA2) in saliva, lung fluids, gastrointestinal secretions, tears, breast milk and vaginal secretions [83].

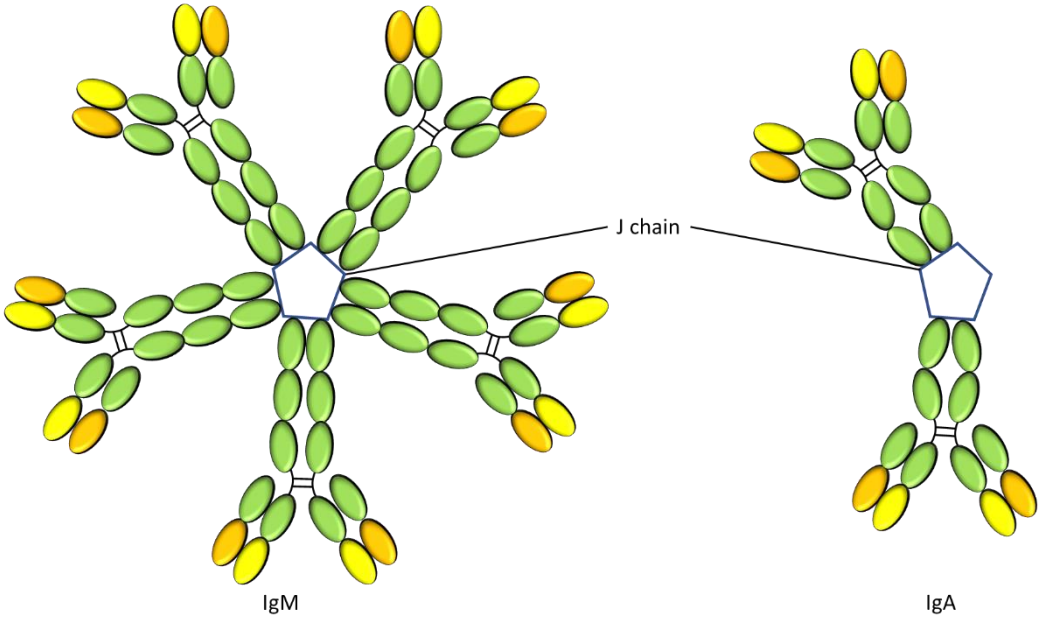

Figure I. 9. The structure of the IgM pentamer and IgA. Adapted from Ref. [83].

IgM has a μ chain and is a pentamer composed by five IgM monomers that are joined by a J chain (Fig. I.9.). Monomeric IgM molecules have the same basic structure as the other immunoglobulins molecules. It is the first immunoglobulin molecule synthesized, making part of the so called primary response. As a pentamer it owns multiple functional domains that can bind to the antigens. However, as the first antibody to be produces during immune response, many IgM do not present the same high affinity to a specific antigen as the later antibodies [83].

IgD has a δ chain, is the least characterized immunoglobulin and its serum concentrations are low [83]. IgD function is still not well understood [90].

IgE has a ε chain and is the largest immunoglobulin monomer, having four C_H domains. IgE levels rise in the response to parasitic infection and in individuals with allergic disease [83].

Table I. 4. Physical properties of immunoglobulins. Adapted form [83].

	IgG	IgA	IgM	IgD	IgE
STRUCTURAL FORM	Monomer	Monomer (circulating) and dimer (secretory)	pentamer	Monomer	Monomer
ACCESSORY CHAINS		J chain (secretory)	J chain		
SUBCLASSES	IgG1, IgG2, IgG3, IgG4	IgA1 and IgA2			
HEAVY CHAIN	γ	α	μ	δ	ε
NUMBER OF DOMAINS IN THE HEAVY CHAIN	3	3	4	3	4
MOLECULAR MASS (kDa)	150	160 (monomer) 385 (secretory)	950	180	190
HALF-LIFE (DAYS)	23	6	5	3	3

I.2.2.2.3. Antibody formats

The versatility of antibodies is further enhanced by the large number of formats available. Antibodies can be monoclonal and polyclonal and they can also be used as intact molecules or as one of several types of fragments. These possibilities make antibodies adaptable to a wide range of assays [91].

Monoclonal vs. Polyclonal Antibodies

Antigens are complex and they present numerous epitopes that are recognized by a large number of B cells. When different lineages of B cell are activated to secrete antibodies against a epitope, the resulting antibody response is polyclonal[92]. In this way, polyclonal antibodies (pAb) are a mixture of different immunoglobulins that can react against a specific epitope of interest [91,92]. In contrast, monoclonal antibodies (mAbs) are antibodies produced by a single B cell clone and they usually present high affinity for one specific epitope [91,92].

As was already mentioned, the antibody-antigen interaction is influenced by conformational determinants, in this way, antibodies may not bind when the antigenic protein is in a denatured state. This characteristic is particularly true for mAbs, which target a single epitope. The impact of conformational change is of less concern when using polyclonal antibodies. pAbs recognize multiple epitopes, some of which are likely to be linear, and conformational changes may not influence all epitopes to the same degree [92].

Additionally, antibodies are able to recognize a relatively small component of an antigen, leading to the possibility of cross-reaction with similar epitopes on other antigens, but usually with less affinity. This fact can be a useful research tool since it can serve as the basis for identifying related antigens. Antibodies with high affinity and specificity for an antigen would present lower cross-reactivity. In this case monoclonal antibodies are more likely to present less cross-reactions [92]. However, while mAbs, are generally preferred because of the its high affinity for the target, pAbs can be generated much more rapidly, at less expense, and with less technical skills, that are required to produce mAbs [91,92]. pAbs are also more stable over a broad pH and salt concentration, whereas mAbs can be highly susceptible to small changes in both. Modification of antibodies by covalently linking a fluorochrome or radionuclide may also alter antibody binding. This is not so concerning when using pAbs, but it can be significant for mAbs if the change affects its monospecific binding site [92]. Thus, the decision of using pAbs, which are relatively easy to produce in a timely and cost-efficient way, or to develop mAbs, which are homogeneous and available in a limitless supply, despite being time consuming and expensive to generate, frequently depends on the application in which the antibody will be used [92].

Antibody Engineering

The high affinity and specificity of mAbs has led to its use in therapeutics as alternative to conventional molecule approaches, being sometimes designed to function by simple binding and neutralization of their target protein [93]. Currently, antibodies can be used in a number of ways, ranging from the intact molecule to a number of different fragments [91] (Figure I.10.). These antibody fragments can be produced by gene technology, where the genes encoding antibody variable domains can be derived from hybridomas or from filamentous bacteriophage displaying antibody fragments [94].

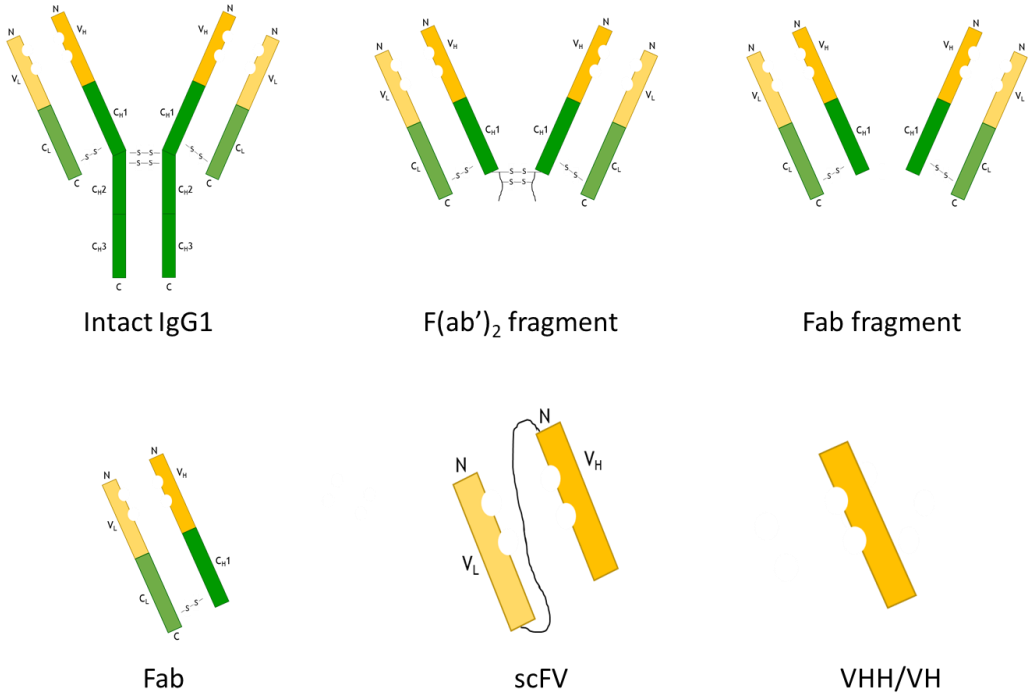

Figure I. 10. Schematic representation of different antibody fragments types. Adapted from Ref. [95].

Nowadays, antibody fragment technology is manly based on three approaches: 1) antigen-binding fragments (Fab); 2) single chain variable fragments (scFv), consisting of the V_H and V_L domains, is the smallest fragment that maintains the full binding capacity of the intact antibody , and 3) V_H domains (VH) [95] (figure I.10.). However, despite the fact that antibodies fragmentation being easier and less costly to manufacture due to the lack of glycosylation and relatively small size, which permits use of prokaryotic expression systems, it may results in altered physiochemical antibody features, leading to reduced shelf life and reduced affinity for their target [95].

The expression of antibody fragments has so far not had a great impact on immunoassay technology. Nevertheless, the possibilities in antibody engineering are, tremendous and will have an effect on the development of antibodies for immunoassays. The principle of shuffling gene segments encoding individual or combinations of complementarity-determining regions (CDR) or entire variable (V) regions creates a variability that can be used for affinity improvements [96]. In this way, these technologies can be useful for enhancing qualitative and quantitative diagnostic parameters, such as increased assay sensitivity; decreased cross reactivity; standardized manufacturing and introduction of novel labeling agents [96].

I.2.3. Immunoassay Systems

I.2.3.1 Immunofluorescence assays

Immunofluorescence (IF) is a common laboratory technique that relies on the use of antibodies which have been chemically conjugated to fluorescent dyes (also called fluorophores or fluorochromes) to label a specific target antigen. The fluorophore allows visualization of the target distribution in the sample under a fluorescent microscope (eg.: epifluorescence and confocal microscopes). The IF technique not only allows to identify the presence but also the distribution of target molecules in a sample. This technique has many different biological applications including evaluation of cells in suspension, cultured cells, tissue, beads and in microarrays. The main limitation of this technique has been the time-consuming process of slide manipulation [97].

There are two classes of IF techniques, depending on whether the fluorophore is conjugated to the primary or the secondary antibody, this method can be direct or indirect (figure I.11.).

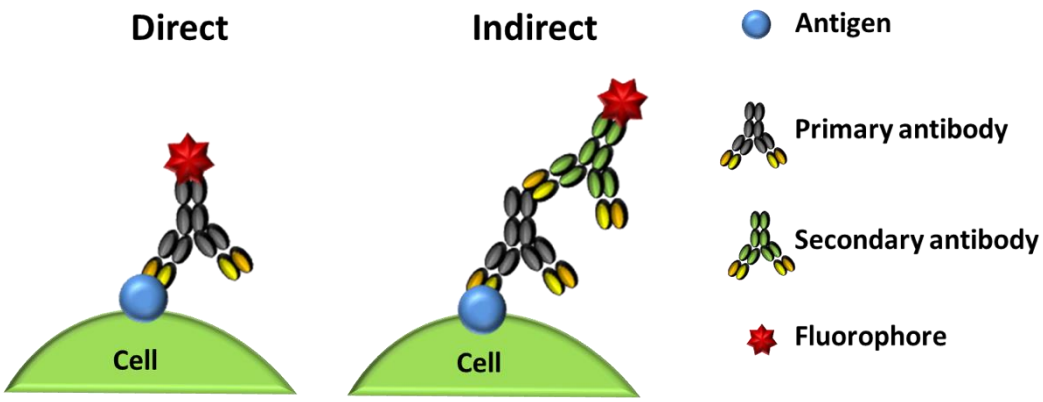

Figure I. 11. Direct and indirect immunofluorescence assay scheme.

Direct IF uses a single antibody that is covalently coupled with a fluorochorme. The antibody recognizes the target molecule and binds to it, and then, the fluorophore allows its detection. This technique has several advantages over the indirect protocol due to the direct conjugation of the antibody to the fluorophore. This reduces the number of steps in the staining procedure making the process faster and can reduce background signal by avoiding some issues with antibody cross-reactivity or non-specificity. However, since the number of fluorescent molecules that can be bound to the primary antibody is limited, direct immunofluorescence is less sensitive than indirect immunofluorescence [80,98].

Indirect IF uses two antibodies: first the unlabeled (primary) antibody which specifically binds the target molecule, and the secondary antibody, which carries the fluorophore, recognizes the primary antibody and binds to it. Multiple secondary antibodies can bind a single primary antibody. This provides signal amplification by increasing the number of fluorophore molecules per antigen. In this way, the indirect technique usually gives brighter fluorescence. This protocol is more complex and time consuming than the direct one, but it allows more flexibility because a variety of different secondary antibodies and detection techniques can be used for a given primary antibody [80,98]. A disadvantage of the indirect technique is that the anti-immunoglobulin sandwich reagent is unable to distinguish between exogenous and endogenous immunoglobulins leading to the possibility of false positive [80].

I.2.3.2. Radioimmunoassay

Radioimmunoassay (RIA) is a highly sensitive immunoassay that measures the concentration of antigen in a sample. In this assay, a quantity of the antigen of interest is tagged with a radioactive isotope and mixed with a known amount of its corresponding antibody. Then the sample is added and the antigens of the sample will compete to bind to the antibody. Bound and unbound antigen are separated, and the amount of radioactivity in the unbound fraction measured. The level of radioactivity in this fraction is proportional to the amount of antigen in the sample [99].

RIA is extremely sensitive, specific and reliable assay that lends itself well to automation. However, the levels of radioactivity during the preparation of labelled reagents are potentially dangerous, being require high qualified personal. Quite apart from the actual risks of isotopes are the legislative requirements, which can increase the costs to a laboratory, in terms of safety measures. These disadvantages argue against its use in small laboratories [76,100].

I.2.3.3. Enzyme immunoassays

Serological methods are playing an important role in the diagnosis and epidemiological assessment of diseases. With regard to immunoassays, enzyme-linked immunosorbent assay (ELISA) has been seen as the basis of the modern immunodiagnostic tests. ELISA is a plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. The detection strategy is accomplished by employing antibodies or antigens conjugated to enzyme, that generates the measurable signal via incubation with a substrate [6,76,101,102].

Enzymes present the advantages of being highly specific, their catalytic properties can enhance a non-enzymatic reaction and the use of various enzyme-amplification circuits can achieve signal levels two to three orders of magnitude greater than those obtained using radionuclides [102]. In this way, ELISA can be as sensitive as radioimmunoassays, and can also be adapted in screening procedures [78,86,103]. Additionally, the reagents present no health hazards, are stable, and have long shelf-lives. Moreover, the estimation of results can either be visual or be made with a rather simple spectrophotometer of the type found in most laboratories [76]. Several enzymes have been used as signal generators, however, just three are widely used, *Horseradish peroxidase* (HRP), *Alkaline phosphatase* (AP), and *β-galactosidase* (βgal). All of these three enzymes possess desirable qualities: their catalysis of highly sensitive reactions, availability of sensitive substrates, adaptability of substrate systems to further amplification, enzyme stability and easy conjugation to antibodies and other proteins[102].

ELISAs are typically performed in 96-well polystyrene plates, where antibodies and proteins passively bind. Having the reactants of the ELISA immobilized to the microplate surface makes it is easy to separate bound from non-bound material during the assay. Also, ELISA can be applied by using various configurations. These configurations are composed of three components: the capture system, the analyte (that can be either an antigen or an antibody) and the detection system. The analyte must be the one in limiting amount; both the solid-phase capture reagent and the detection system must be present in functional molar excess [102].

For direct ELISA method the sera containing the target antigen is coupled to a solid-phase support and then an antibody labelled with an enzyme (conjugate) is added against to the target antigen (Figure I.12.). Direct ELISA detection is much faster than other ELISA techniques as fewer steps are required. The assay is also less susceptible to error since fewer reagents and steps are needed, i.e. no potentially cross-reacting secondary antibody needed. Although there are also some disadvantages as the antigen immobilization is not specific, higher background noise may be observed in comparison to indirect ELISA because all proteins in the sample, including the target protein, will bind to the plate. Direct ELISA is less flexible since a specific conjugated primary antibody is needed for each target protein. As no secondary antibody is used there is no signal amplification, which reduces assay sensitivity. Finally, the direct ELISA technique is typically used when the immune response to an antigen needs to be analyzed.

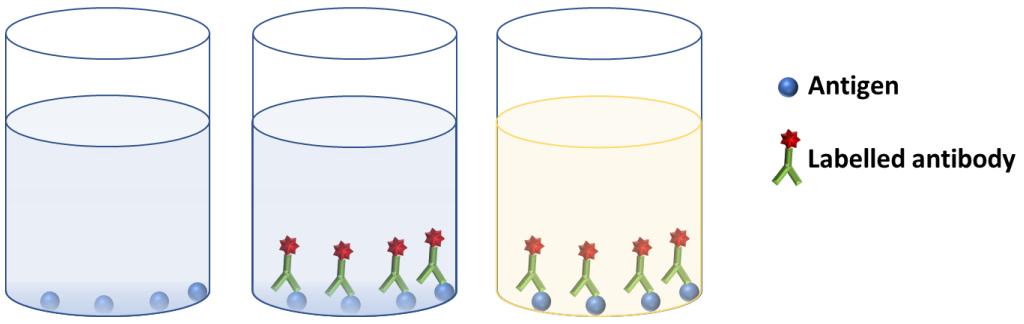

Figure I. 12. Schematic representation of direct ELISA.

For indirect ELISA method (figure I.13.), the antigen is coupled to a solid-phase support and the sera, containing the target antibody, are incubated in this system. Then a secondary antibody labelled with an enzyme (conjugate) is added against to the target antibody. The conjugate will become attached to the system, and the amount of conjugate attached is measured by the amount of substrate that it degrades. This is a very useful method, since a single enzyme-labelled antibody can be used to detect human antibodies [78,103].This method has high sensitivity since more than one labeled secondary antibody can bind the primary antibody. Indirect ELISA delivers greater flexibility since different primary antibodies can be used with a single labeled secondary antibody. Among its disadvantages is the possibility of cross-reactivity of secondary antibody to the adsorbed antigen, which could increase background noise. Also, indirect ELISA assays take longer to run than direct ELISAs since an additional incubation step for the secondary antibody is required. The indirect ELISA is most suitable for determining total antibody concentration in samples.

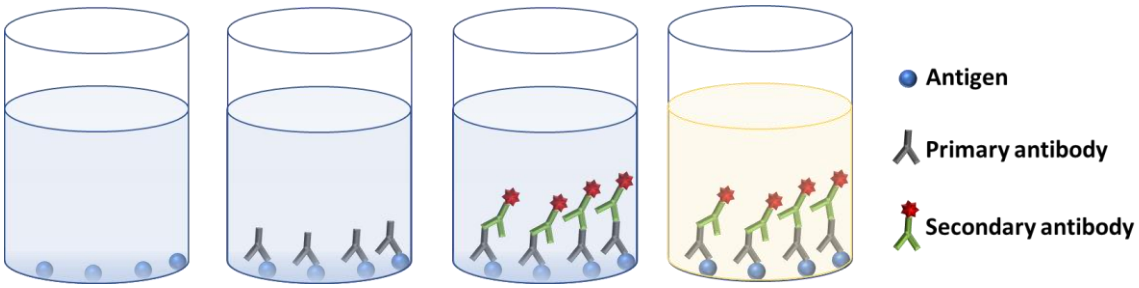

Figure I. 13. Schematic representation of indirect ELISA.

For the sandwich method a capture antibody is used to be immobilized on the solid surface (Figure I.14.). The solution containing the antigen is then added, followed by the detection antibody. The detection antibody can be enzyme conjugated, in which case this is referred to as a direct sandwich ELISA. If the detection antibody used is unlabeled, a secondary enzyme-conjugated detection antibody is required. This is known as an indirect sandwich ELISA [78,86].

This type of capture assay is called a “sandwich” assay because the analyte to be measured is bound between the capture antibody and the detection antibody [78,104].

The major advantage of a sandwich ELISA is its high sensitivity. Sandwich ELISA also delivers high specificity as two antibodies are used to detect the antigen and also offers flexibility since both direct and indirect methods can be used. Nevertheless, there are few disadvantages, if a standardized ELISA kit or tested antibody pair is not available, antibody optimization is required in order to reduce cross-reactivity between the capture and detection antibodies. Sandwich ELISAs are particularly suited to the analysis of complex samples, since the antigen does not need to be purified prior to the assay [103].

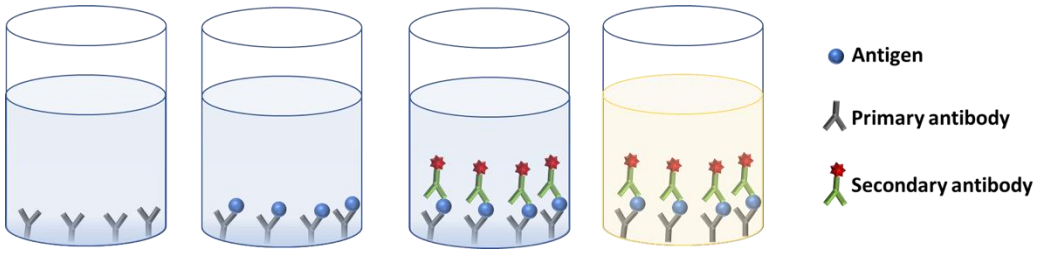

Figure I. 14. Schematic representation of sandwich ELISA.

The competitive ELISA, also known as a blocking ELISA, is perhaps the most complex of all the ELISA techniques. However, each of the above assay types can be adapted to a competitive format. For the competitive method, a specific antibody is attached to the solid surface. Then, the sample containing the antigen is added and followed by the enzyme-labelled antigen. The amount of enzyme-labelled antigen attached is measured by the rate of hydrolysis of its substrate. The more antigen there is in the unknown solution, the less labelled antigen will be attached. The higher the sample antigen concentration, the weaker the output signal, indicating that the signal output inversely correlates with the amount of antigen in the sample [78,86].

In all these methods the end result is a change in color of the enzyme substrate. This can be measured accurately in a spectrophotometer [78,86,103].

I.2.3.4. Alternative immunoassays

Frequently, the term 'biosensor' or 'immunosensor' is used to label an immunoassay system that is an alternative to a conventional assay system, developed with automated and fast, possibly direct, data acquisition as a requirement. Some alternative immunoassays configurations will be discussed below.

The first one is surface plasmon resonance (SPR), which offers a flow-through biosensing technology that measures very small changes in refractive index due to the generation of surface plasmons on a noble metal surface when mass binds to that surface. To conduct a typical antibody-antigen binding study, the antibody is immobilized on a thin gold film. When the antigen from the solution binds to the immobilized antibody, the binding will cause a refractive index change at the surface layer, which is detected by SPR through resonance angle change of the reflected light. However, the sensitivity of this method has to be improved and is not suitable for a screening test [88].

Conventional solid-phase immunoassay techniques are known to present time-consuming procedures because the antigen-antibody interaction occurs at a solid phase surface. Flow-injection principles can be used to enhance the efficiency of the immune-interaction. In

this case, the immunoassay system is arranged as a column containing immobilized antibodies. The column is saturated with a solution containing the labeled antigen. After antigen-antibody interaction has occurred, is injected the unlabeled antigen in the column. The affinity of antibodies for labeled antigens is usually significantly lower than their affinity for unlabeled (free) ones owing to stearic factors. Therefore, injection of free antigen into the column results in displacement of the labeled antigen by the unlabeled antigen. Labeled antigen is then detected at the outlet of the column. A similar scheme can be realized based on the use of immobilized antigen. The analysis time in this immunoassay is normally several minutes. However, the method's sensitivity and the level of background signal can be especially critical and the displacement of labeled antigen by free antigen is difficult. Only a high concentration of free antigen will be able to displace labeled antigen in amounts suitable for detection. Additionally, for each particular analyte, definite conditions (including column, flow rate, buffer solution) have to be specially optimized [75].

Electrochemical immunosensors produce electrical signals for the quantitative analysis of target molecules. Electrochemical detection methods possess high sensitivity, low cost, low power requirement, and high compatibility with advanced micromachining technologies. They have been extensively applied in immunosensing and meet the need for the development of point of care (PoC) systems [77,105]. Electrochemical immunosensors will be discussed in more detail in section 3 of this chapter.

I.3. Biosensors

I.3.1. Biosensor concept

The ability to detect pathogenic agents and molecules with physiological interest in the body, with high sensitivity and specificity, by using low cost technologies, allows the early diagnosis and treatment of diseases, reducing at the same time the costs associated with the care of advanced stages patient [106]. Clearly, there is a need for rapid, cheap, sensitive, specific and reproducible technologies, compared to established techniques that are typically cumbersome and/or expensive to be applied routinely [107].

From the time when Clark and Lyons developed the first glucose oxidase sensor to detect blood glucose, in 1969 [108], biosensing technologies have expanded and suffered many improvements in their sensitivity and selectivity [107,109]. Nowadays, a biosensor can be defined as an analytical device, which comprises three components: 1) a biological recognition system (such as enzymes, antibodies, tissue, DNA, cells and organelles) that has the ability to recognize the target analyte with high specificity, 2) a transducer which measures the changes in physical-chemical properties of the biological recognition system during the interaction and transfers the signal to a useful output and 3) the output system which involves the amplification and displays the signal in an appropriate format [106,109-113]. Figure I.15. shows a schematic representation of a biosensor.

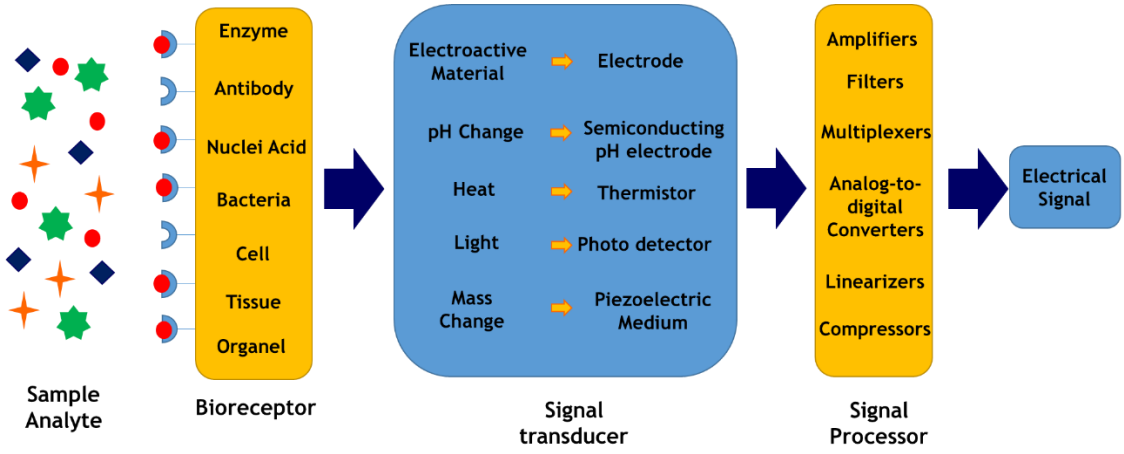

Figure I. 15. Schematic representation of a biosensor. Adapted from Ref. [78],[110] and [115].

Biosensors can be classified according two different parameters: the type of biological recognition system and the type of the signal transducer [111,115,116]. The choice of the biological material and the adjusted transducer depends on the properties of each sample of interest and the type of physical magnitude to be measured [116]. The transducer can take many forms, but the electronic configuration has gained great importance.

I.3.2. Biological recognition system

The type of the biocomponent determines the biosensor degree of selectivity or specificity and can be classified in three groups: biocatalytic, bioaffinity and micro based receptors [116]. The biocatalytic group comprises enzymes and tissue, the bioaffinity group includes antibodies and nucleic acids, and the micro-based group is represented by microorganisms [116].

The biocatalytic-based biosensors are the best known and studied and have been most frequently applied as biological matrices. In this group, enzyme based biosensors present the highest sensitivity and selectivity when compared to the use of plant or animal tissues [116,117].

Enzyme based sensor were the earliest biosensors to be developed, and since then, they have been facing a massive growth in usage for various applications. Enzymes are very efficient biocatalysts, which have the ability to specifically recognize their substrates and to catalyze their transformation. These unique properties make the enzymes powerful tools to develop analytical devices [109,117]. In biosensors, enzymes are generally immobilized on or close to the transducer. The use of enzymes with specificity for a limited range of substrates enables greater selectivity [109,117]. Enzymes own six major classes: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. Oxidoreductases catalyze oxidoreduction reactions; transferases catalyze a group transfer from a donor to an acceptor; hydrolases catalyze hydrolytic cleavage of C–O, C–N, O–P and C–S bonds. These enzymes can be considered as a special group of transferases since the reaction catalyzed is the transfer of a specific group on water. Lyases are enzymes catalyzing the nonhydrolytic cleavage of C–C, C–O, C–N, and other bonds by elimination, leaving double bonds or rings, or conversely the addition of groups to double bonds; isomerases catalyze isomerization reactions; and ligases catalyze the formation of new bonds between two molecules [118]. Nevertheless, the most commonly used enzymes in biosensing are the oxidoreductases [116]. Finally, is important to remember that due to their protein nature, enzymes are often fragile and this instability results in a decrease in the enzyme activity and consequently in a decrease in the biosensor performance [118].

Tissues for tissue-based sensors arise from plant and animal sources [116]. These sensors use tissue slices as sources of enzymes that catalyzes a specific reaction. They present the advantage of being highly stable leading to a high level of enzymatic activity resulting from the maintenance of the enzyme in its natural environment. This high stability is also responsible for biosensors long self-life. Additionally, tedious and time-consuming procedures for enzymes extraction and purification are avoided [119]. However, they suffer from long response times, due to the diffusion barriers, and low specificity, as already mentioned, due to the presence of other enzymes in the tissue [116,119].

Immunosensors, included in the bioaffinity group, were established on the fact that antibodies have high affinity towards their respective antigens, as they specifically bind to pathogens or toxins, or interact with components of the host's immune system [116]. In practice,

this type of biosensors present the advantage of being highly sensitive and selective to the detection and quantification of trace substances [117]. There are many ways to implement immunoassays based on the signal transducer, label type, and assay format used. Immunoassays, can also 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 [117]. When we are coupling the immunoreaction to an electrochemical transducer, it is usually required the use of a labeled immunoagent [120].Once antibodies are not electrochemically actives and cannot participate directly in redox reactions. Enzymes and nanoparticles, are often used as labels, as they facilitate production of electroactive species[120].

The highly specific affinity binding's reaction between two single strand DNA chains to form double stranded DNA is used in the nucleic acids based biosensors, which also includes this type of biosensors in the bioaffinity category [109] . In biosensors based on DNA, a single strand DNA is able to recognize and bind to its complementary strand in a sample, by the formation of stable hydrogen bonds between the two nucleic acid strands[116]. The pairing of the both DNA strands, or hybridization, is commonly detected via the increased current signal of an electroactive indicator (that preferentially binds to the DNA duplex), in connection to the use of enzyme labels or redox labels. Nevertheless, despite the fact that nucleic acids being relatively simple molecules, finding the sequence that contains the desired information and the distinction between perfect matches and mismatches, are very challenging tasks [121]. Aptamers are artificial affinity probes of nucleic acid (DNA or RNA), selected through combinatorial libraries to bind specifically target molecules. The remarkable target diversity, tight-binding capability, ease-of synthesis and high stability of aptamers hold great promise for biosensing [122,123].They are also powerful analytical tools because of its high selectivity and high sensitivity, however they usually require complicated chemical labeling and assembling procedures [124].

Cell based sensors are a type of biosensor, included in the micro-based group, which use living cells as biospecific sensing elements. Microorganisms such as bacteria and fungi can be used as biosensors to detect specific molecules of the surrounding environment [109]. Microbial sensors are less susceptible to the inhibition by other compounds present in the sample, are more tolerant to the pH and temperature variations, and generally have longer lifetime being easily regenerated, by immersion in a solution of nutrients. Nevertheless, they normally present a slow response and low selectivity due to a variety of metabolic processes occurring in an living cell [116,117].

I.3.2.1. Immobilization of the sensitive element

The biologic recognition system immobilization step is critical in the development of any sort of biosensor. It provides the core of the biosensor and gives it its identity [125]. Moreover, the immobilized biomolecule needs to keep its original functionality as longer as possible in

order to maintain the biosensor performance [126]. This means that care must be taken so that the recognition sites are not sterically hindered [125,126]. Another common reason for biosensor failure or underperformance is the chemical inactivation of the active/recognition sites during the immobilization stages. There is no universal immobilization method suitable for every application imaginable. When it comes to choosing the immobilization method, there are other important factors that need careful consideration, such as the type of transduction used, the nature and composition of the sample and the possibility of multiple use of the biosensor [125]. The following procedures are the most generally employed (figure I.16.).

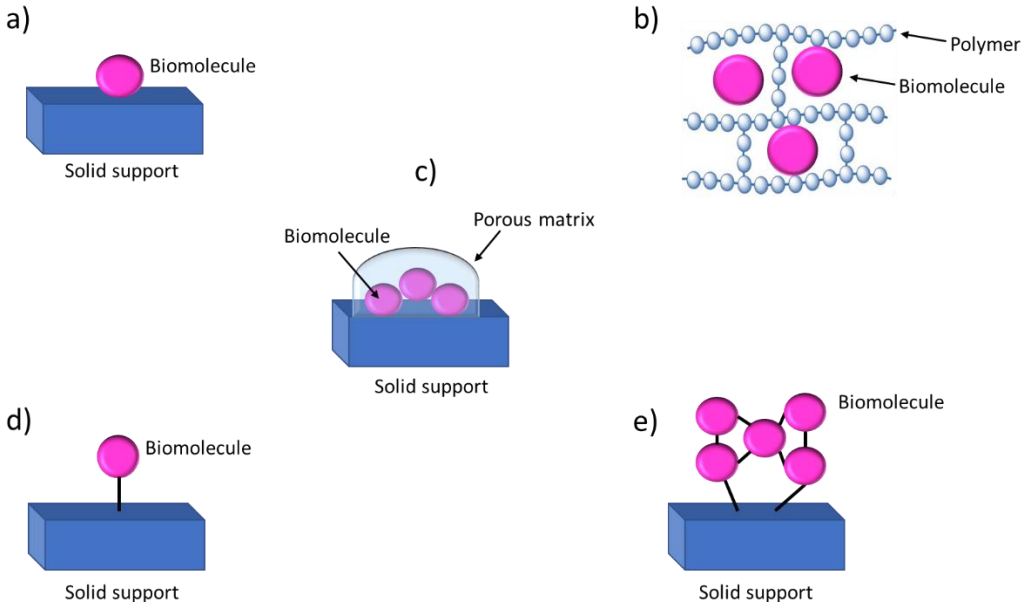

Figure I. 16. Different immobilization methods a) Adsorption; b) entrapment; c) encapsulation; d) covalent binding and e) cross-liking. Adapted from Ref. [114].

The easiest, simple and quickest approach is to physically adsorb the biomolecule on the electrode surface. Physical adsorption (figure I.16.a)) is based on van der Waals, ionic bonds, electrostatic and hydrophobic interactions between biomolecule and solid sensor surface [127]. In this type of immobilization mild conditions are used, which are less disruptive to biomolecule. However, biomolecule linkage is highly dependent on pH, solvent and temperature and the adsorption occurs randomly, which does not guarantee the proper immobilization [127]. Additionally, this method does not employee strong binding forces and the attached biomolecules may be released or not persist on the sensor surface [114].

Entrapment (figure I.16.b)) is another physical immobilization method, where the biomolecule is entrapped inside of an adequate matrix. Within this matrix the biomolecule stability is improved. Moreover, immobilization in matrices like gels, polymers, pastes or inks can be as simple as physical adsorption. Generally, the biological material is mixed and well homogenized with the supporting material and then applied over the electrode pad as an additional membrane that has to be dried or polymerized [128]. Some of the matrices used are

gelatins, polyurethanes [128]. This method carries large diffusional barriers and loss of biomolecule activity [127].

In encapsulation method (figure I.16.c)), a porous matrix is formed around the biological material which helps binding onto the sensor [114]. One approach for encapsulation uses sol—gel method for the immobilization of biological molecules in ceramics, glasses, and other inorganic materials. These matrices allow optical monitoring of the chemical interactions since they are optically transparent. The sol—gel process can be performed at room temperature, protecting biomolecules against denaturation. Biomolecules immobilized by this procedure are very stable, but is difficult to achieve sol—gels reproducible pore sizes. Problems such as diffusional limitations inside the porous network, brittleness of the matrix, reproducibility and complicated procedure are the disadvantages of this method [114].

For covalent bonding (figure I.16.d)) the sensor surface is modified to acquire a reactive group to which the biological materials can be attached. Usually, nucleophilic functional groups present in amino acid side chains of proteins such as amino, carboxylic, imidazole, thiol, hydroxyl etc. are used for coupling [114]. This method improves uniformity, density and distribution of the bioelements, as well as reproducibility and homogeneity of the surfaces [114]. Covalent immobilization may decrease or eliminate some common problems such as instability, diffusion and aggregation. For this purposes the reagents such as glutaraldehyde, carbodiimide, succinimide esters, maleinimides and periodate are often used for covalent immobilization [114]. Nevertheless, the procedures are usually complicated and time-consuming, existing also the possibility of biomolecule activity losses [127].

Cross-linking procedure (figure I.16.e)) is a covalent immobilization method configuration wherein is used bi-functional or multi-functional reagents such as glutaraldehyde which couples with the lysine amino groups [114]. In this method occurs the formation of three-dimensional links between the biomolecule and the bi or multi-functional reagent. The result of cross-linking are biomolecules with irreversible intermolecular bonds capable of withstanding extreme conditions of pH and temperature. The cross-linking procedure is simple and avoid losses of the biomolecule activity due to diffusion effects, Nevertheless, it requires a large amount of biomolecules [127].

I.3.3. Transducer

The purpose of the transducer is to convert the biochemical reaction into a signal that can be suitably processed as an output [117]. Taking into account the principle on which its operation is based, the transducers can be categorized as: electrochemical, optical, calorimetric and piezoelectric. Transducer's choice depends on the properties of the sample of interest and the type of physical magnitude to be measured [116,117].

In the biosensors based on an electrochemical transducer, the chemical reaction between the immobilized biomolecule and the target analyte produce or consume ions or

electrons, which affects measurable electrical properties of the solution [114]. These electrical properties, can be correlated to the concentration of the electroactive species or its rate of production/consumption. The resulting electrical signal is related to the recognition process of the target analyte, being proportional to its concentration [109]. These biosensors have the advantage of: being simple, reagentless, cost competitive, portable and prone to automation; and of presenting fast responses [109,112,129]. These advantages make them the most commonly used and the most frequently cited types of sensors in the literature, playing a significant role in medicine, agriculture, food safety, bioprocessing and environmental and industrial monitoring once they can detect and provide accurate and reliable information about molecules of analytical significance, pathogens and toxic compounds [120, 116].

The optical biosensors are based on methods such as UV–Vis absorption, bio/chemiluminescence, fluorescence, reflectance, and refractive index measurements, which are used to analyze the interaction of the biocatalyst with the target analyte through alterations of the electromagnetic radiation [114,116]. Nowadays, these biosensors are receiving considerable attention due to the advances in optical fibers and laser technologies [116]. Nevertheless, the interference from ambient light, the requirement of high-energy sources and the fact that miniaturization can affect the magnitude of the signal, are disadvantages associated to this type of biosensors, that cannot be forgotten [116].

Calorimetric biosensors are constructed by immobilization of biomolecules onto heat sensors. Once the analyte comes in contact with the biocomponent, the reaction heat, which is proportional to the analyte concentration is measured [114]. These changes in energy are measured by thermistors. Although, the electrochemical and the optical biosensors are more commonly used, thermal transducers can be sufficiently effective in analytical applications. The use of thermal biosensors in food analysis is still limited, probably due to tradition and the relative complex instrumentation involved. Despite the lack of selectivity, which is a characteristic problem of these type of transducers, they present advantages of miniaturization and the possibility of construction of sensors arrays for the simultaneous determination of several compounds [116].

Piezoelectric biosensors are based on the coupling of a bioelement with a piezoelectric component, usually a quartz-crystal coated with gold [114]. The interaction of this biological recognition element with the analyte can be monitored through the oscillation of the crystal immersed in a liquid, which will produce a modification of mass in the crystal, perceptible by means of its frequency of oscillation[116]. Piezoelectric transducers allow label-free detection of molecules, being for this reason most applied in immunosensors [114,116], however they present a low sensitivity [116].

I.3.3.1.Electrochemical transductors

Nowadays, as already mentioned, the most widely used transducers are those with an electrochemical detection, as they present low cost, are easily handled, can be portable, their construction is simple and give fast responses [130]. In this type of transducers, the bioreaction, which is electrochemically monitored, may generates a measurable charge accumulation or potential (potentiometry), may alters the conductive properties of the medium between electrodes (conductometry) or may produces a measurable current (amperometry). The use of electrochemical impedance spectroscopy by monitoring both resistance and reactance in the biosensor is also becoming more common [130]. In this way, the electrochemical transducer used in biosensors can be distinguished as: potentiometric, conductometric, impedimetric and amperometric.

In potentiometric measurements, the potential difference between a working and a reference electrode is determined by a voltammeter when there is no significant current flowing through them. The potential difference is measured due to the oxidation and reduction of the species in a sample solution [112]. Challenges to the use of these devices are the lack of sensitivity due to de difficulty in distinguish between concentrations within the same order of magnitude [77].

In conductometric measurements, there is a relationship between a biorecognition event and conductance. While a reaction occurs, a change in the ionic species concentration leads to change the solution's electrical conductivity or current flow. A conductometric biosensor consists of two metal electrodes (usually platinum or silver) separated by a certain distance. An ohmmeter (or multimeter) is used to measure the change in conductance between the metal electrodes [7].

In impedimetric measurements, when biorecognition by the biomolecule occur at the modified surface, the interfacial properties change. Thus, impedimetric biosensors can be used to determine quantitative parameters of electrochemical properties. Electrochemical reactions involve electrolyte resistance, adsorption of electroactive species, charge transfer at the electrode surface, and mass transfer from the bulk solution to the electrode surface. Each reaction process represented by an electric circuit consists of resistance, capacitors, or constant phase elements combined in parallel or in series [7].

Most of the existing work, present in literature, has been carried out with amperometric transducers [116]. The current produced by an electrochemical reaction at an applied potential is measure and related to the concentration of species in solution [116]. Amperometric biosensors are faster, more sensitive, more precise and accurate than the potentiometric ones, for this reason they are the most commonly used [116,130]. Nevertheless, due to the type of electrodes used, in the past these type of biosensors presented low reproducibility[131]. In our days, the development of screen-printed electrodes exceeded this limitation and opened important expectations in the construction of miniaturized biosensors based on amperometric transducers.

I.3.4. Screen-printed electrodes

One of the most common problems of electroanalytical techniques has been their lack of reproducibility, related as mentioned before with the difficulty of obtaining identical electrodes for all measurements [131]. The conventional hanging drop mercury electrode is the one that has presented the least problems in this sense, all the others generally used (platinum, carbon paste and vitrified carbon) do not surpass the up mentioned disadvantage. Still, mercury electrodes are very toxic. Thus, electrochemical techniques need to be improved by means of classical electrodes and cell systems replacement. One option that fits well with the expectations, is the use of disposable screen-printed devices [131].

Screen-printed electrodes (SPE) technology has the advantage of offering high-volume production in an extremely inexpensive way, these electrodes are highly reproducible, susceptible to automation and disposable, avoiding some problems related to traditional solid electrodes such as the need of a cleaning processes. In addition, SPEs are also flexible, since a wide range of designs and materials can be applied in their construction. Also, the fact of being miniaturized devices leads to the reduction of sample volume required, which in turn helps reducing the overall size of the diagnostic system and open the possibility of its application *in situ* [131-134].

For the construction of SPEs a series of basic stages is required, namely selection of the screen, selection and preparation of the inks, selection of the substrate and the printing, drying and curing stages [28]. SPEs usually includes a three electrodes configuration printed on various types of plastic or ceramic substrates, which is easily modifiable with a great variety of inks.

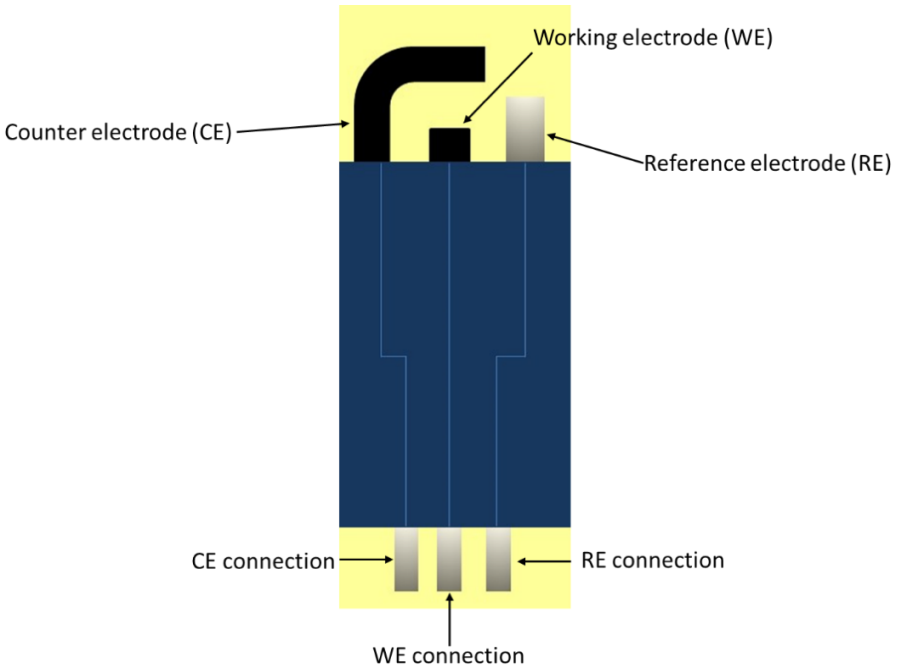

Figure I. 17.Traditional screen-printed electrodes constitution. Adapted from one of screen-printed electrode design made in University of Burgos.

The traditional electrode system contains a working electrode (WE), a reference electrode (RE) and an auxiliary or counter electrode (CE) (Fig. I.17.). The WE is the electrode on which the reaction of interest is occurring, and is where the potential is controlled and where the current is measured, the RE is an electrode which has a stable and well-known electrode potential and a CE is generally an inert conductor (usually made of the same material as the WE) that completes the cell circuit, once the current that flows into the solution via the WE leaves the solution through the CE [135]. Needless to say that the analyte must be an electroactive product, in order to undergo a redox reaction at the electrode [107].

I.3.4.1. Screen printed electrodes fabrication

For the construction of SPE, a sequential layer deposition of different inks on a ceramic or plastic substrate is made by using an appropriate screen. This screen is made from a finely mesh of different materials such as stainless steel, polyester or nylon mounted under tension on a metal frame. There is an open-mesh in a specific area of the screen, through which the desired pattern can be printed (figure I.18. a)). The screen is held in position within a screen-printing machine at a distance of around 0.5 mm from the surface of the substrate [136].

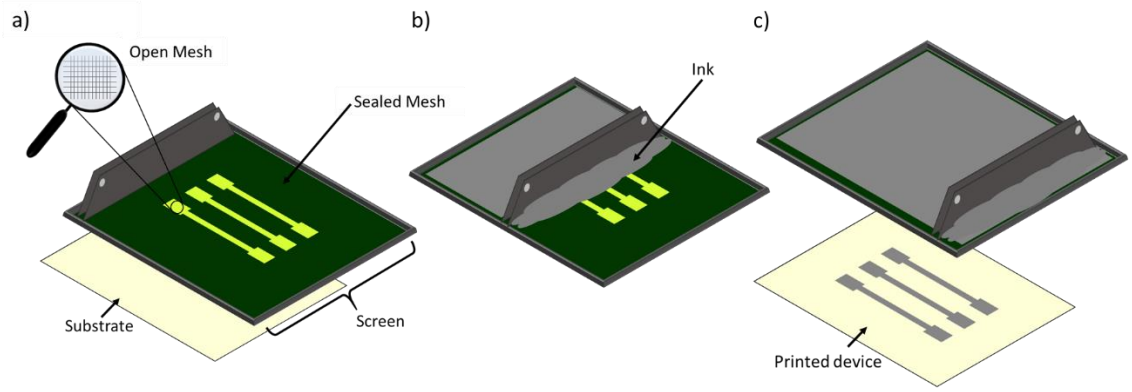

Figure I. 18. Scheme of a screen-printed device fabrication process: a) screen poured; b) Squeegee traversing; c) Ink deposited on the substrate surface. Adapted from Ref. [134].

During the printing process, the ink, which is typically resistive, conductive or dielectric, is placed on the screen surface. Then, a squeegee moves from the back to the front part of the screen, forcing the ink to pass through the open mesh areas of the screen (figure I.18. b)). After this, the required pattern is deposited onto the substrate surface, as it can be seen in figure I.18. c) [136]. The next phase of the process is to dry the printed ink. All inks used

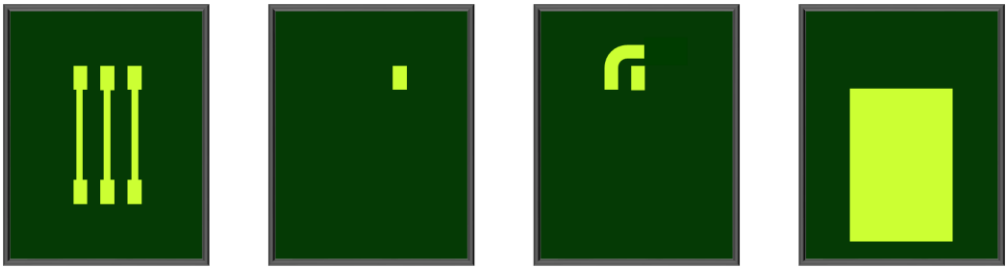

Figure I. 19. Schematic representation of the screens required for the construction of screen-printed electrodes. Adapted from screens used in University of Burgos.

for the fabrication of SPEs contain various organic solvents, which are essential to obtain the correct viscosity for screen printing process. These solvents can be removed by drying the film in an infrared belt drier, or in a conventional box oven, at a temperature of around 150 ºC. After drying, the films retain a rigid pattern on the substrate [136]. The combination of different screens and inks allows the production of different electrodes (working, reference and auxiliary) in the same configuration unit (figure I.19.). Finally, for the assembly of a disposable electrochemical biosensor a modification of the working electrode with a biological recognition element is carried on. This modification will influence the robustness and sensitivity of the developed biosensor [131,132,134,135].

I.3.4.2. Materials used on screen printed electrodes and their modification

The most common screen printed electrode configuration is the one constituted by three types of electrodes: the working electrode, which is usually made of gold, carbon, or platinum; a reference electrode, usually from silver or silver chloride, which has a fixed potential that controls the potential of the working electrode; and the third electrode which is called the counter or auxiliary, which is included sometimes to help measure the current flow [109,133].

Metals and carbon are commonly used to prepare electrode systems and supporting substrates. Metals such platinum, gold, silver and stainless steel are use due to their excellent electrical and mechanical properties. Carbon-based materials, such as graphite, carbon black and carbon fiber are also used to construct the conductive phase. These materials have a high chemical inertness, provide a wide range of anode working potentials with low electrical resistivity and have a pure crystal structure that provides small residual currents and a high signal-noise ratio [134,135]. Additionally, graphite materials are chosen due to their simple technological processing and low-cost [132,134].

Also present in SPEs printing inks are some mineral binders or insulating polymers to improve adhesion onto the substrate. The existence of these polymers in inks for working

electrodes might shelter the electrochemically active carbon particles and increase the electron transfer resistance, resulting in slower kinetics of heterogeneous reaction, and quasi-reversible or irreversible redox processes. In this way, to improve the slow electron transfer of bare SPEs, it is possible to modify the printing paste with certain materials. Currently, noble metals show the best overall catalytic performance and play a dominating role as electrocatalysts [134]. Indeed, noble metal nanoparticles (NPs) have played an important role in the development of new biosensors and/or in the enhancement of existing biosensing techniques to fulfill the demand for more specific and highly sensitive biomolecular diagnostics. NPs are, in general, one of the most common nanotechnology-based approaches for developing biosensors, due to their simplicity, physiochemical malleability, rich electronic and catalytic properties and high surface areas [137]. NPs can measure between 1 to 100 nm in diameter, they can have different shapes and can be composed by noble metals, heavy metals and iron. Noble metal NPs, in particular gold and silver NPs, are among the most extensively studied [137]. These nanoparticles have good conductivity, making them suitable for acting as electronic supports, as they increase the electronic transfer between redox centers in the biomolecule and on electrode surfaces [138,139], functioning as electron-conducting pathways between prosthetic groups and the electrode surface [140]. Furthermore, gold nanoparticles also provide high surface-to-volume ratio, high surface energy, ability to decrease proteins–metal particles distance, and stable immobilization of biomolecules with retention of their bioactivity [140]. On the other hand, nanoparticles of non-noble metals are more difficult to obtain because they oxidize easily both in contact with air and in solution[139].

Another type of nanostructures particularly attractive for bioelectronic detection are the carbon nanotubes (CNTs) used also to monitoring biorecognition events and biocatalytic processes [129]. NTs are excellent electrode materials due to their unique structure, good electrical conductivity, high chemical and thermal stability, high mechanical strength and large surface area [141,142]. As in the case of NPs, because of the high surface-to-volume ratio and novel electron transport properties of these nanostructures, their electronic conductance is strongly influenced by minor surface perturbations (such as those associated with the binding of macromolecules), allowing sensitive label-free bioelectronic detection [137]. CNT can be divided into single-wall carbon-nanotubes (SWCNT) and multi-wall carbon-nanotubes (MWCNT). SWCNT possess a cylindrical nanostructure, formed by rolling up a single graphite sheet into a tube. MWCNT comprise several SWCNT that are concentrically nested like rings of a tree trunk [137].

Finally, quantum dots (QD) or semiconductor nanocrystals (CdS, PbS, and ZnS) have been used on electrochemical biosensors due to their intrinsic redox properties, which allows the electrochemical signal amplification [142-144]. Additionally, they also present biocompatibility, being suitable for biomolecules immobilization and increase the surface-to-volume ratio [145].

I.3.5. Electrochemical immunosensors

Immunosensors are antibody–antigen based affinity biosensors, in which the detection of antigen as a target analyte is a result of the specific binding of the antigen to particular region of an antibody on the electrode surface. Antibody acts as a bioreceptor and antigen acts as a target analyte and the transducer is then able to quantify the antigen concentration[112,146] .

Electrochemical immunosensors can present different sensing mechanisms, as for conventional immunoassay, they can present the sandwich and competitive configuration. Sandwich immunosensors are currently the most commonly and successfully used, mainly due to their high sensitivity and minimized background signal. Sandwich immunosensors can be performed on any kind of sensing surface. The main criterion of these assays is the availability of two antibodies with different binding sites on the antigens. In a competitive immunoassay, the labeled analyte competes with the analyte for the antigen binding sites on the antibody. The key feature of a competitive assay is that maximal assay sensitivity is attained when the signal tends to zero [104]. This strategy is useful to find the suitable inhibitory molecules that can have better affinity for the antibody [86].

Electrochemical immunosensors have attracted considerable interest because of their high sensitivity, low cost, and inherent miniaturization [144]. These type of immunosensors are portable, simple, easy to use and disposable in most cases. Thus, compared with traditional immunoassay methods, electrochemical immunosensors are specific, simple and convenient, they also can offer multitarget analyses and perform *in situ*, real-time automated detections [112,147]. Additionally, the specific binding of an antibody to its target antigen in a complex mixture such as serum and plasma provides the detection and quantification of diseases at levels as low as the picogram [112].

I.3.5.1. Antibody immobilization and its monitoring

Antibody immobilization on a solid support is an essential process for the development of an immunoassay. When antibodies are directly conjugated to the surface, antigen detection is often interfered by steric hindrance and the limited mobility of bound antibodies. The critical problem, during the immobilization process, is the possibility of antibody denaturation or the occurrence of conformational changes. Antibody modification, especially on antigen binding sites, is one of the factors that can lead to a reduced immunosensor sensitivity [148–151].

Immobilized IgG can adopt four molecular orientations: 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) (Figure I.20.). For the highest analyte binding, antibodies should display the highest free antigen-binding regions after

immobilization [151]. In this way, properly oriented antibodies, with their antigen binding sites well exposed to the solution containing the analyte, exhibit higher antigen binding capacities compared to randomly oriented antibodies. Additionally, uniformly oriented immobilization also provides high consistency for the construction of immunosensors. For these reasons, the oriented immobilization of antibodies has become essential to optimize antigen detection during the development of an immunoassay (figure. I.20.) [148].

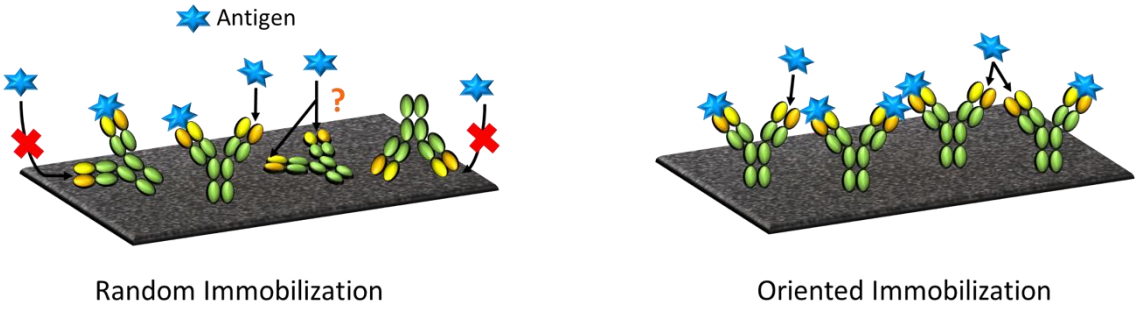

Figure I. 20. Schematic representation on antibody immobilization. a) random immobilization. b) oriented immobilization. Adapted from Ref. [148,151].

There are several possibilities for the immobilization of the antibody. Immobilization of an antibody by adsorption on a solid surface is the simplest method because it does not require the use of multiple materials and complex reactions [150]. Adsorption occurs by physical interactions, avoiding an intermediate protein. Adsorption make use of ionic bonds, electrostatic and hydrophobic interactions and van der Waals forces between antibodies and the solid substrates. This method present low control over antibody orientation, leading to random immobilization [148,151]. Typically, biosensors using this immobilization method suffer from poor analytical performance and storage stability [151].

Alternatively, antibodies can be covalently coupled to chemically activated surfaces. In this approach, amino groups on the antibody surface can be readily coupled with several reactive moieties, such as aldehyde, epoxy, and N-hydroxysuccinimide (NHS) on various solid surfaces[148]. A popular covalent antibody immobilization method is based on the streptavidin-biotin interaction[151]. Antibodies can be biotinylate using NHS-activated biotin and then immobilized onto streptavidin-coated surfaces [149]. Although chemical immobilization is a highly stable process, covalently-linked antibodies through amino groups are also randomly oriented, which can cause some of the immobilized protein to lose binding activity due to direct chemical modification of the antigen binding site, steric hindrance by the surface itself, steric hindrance by adjacently immobilized antibodies, and denaturation due to strain from multiple attachment sites. For these reason, antibodies are sometimes specifically immobilized onto surfaces such that the antigen-binding site is oriented away from the surface itself [148,149].

For antibody specific orientation during the immobilization procedure, it is required the use of an intermediate protein, itself directly coupled to the surface, that binds to the Fc region of antibodies. Antibody-binding proteins such as protein G and protein A specifically target the Fc region [149]. Proteins A and G are biomolecules characteristic to certain species of

pathogenic staphylococcal and streptococcal bacteria. Protein G, in its native form, is expressed in *Streptococci* and it recognizes both Fab and Fc domains of immunoglobulins. It binds to all human, rabbit, mouse, and goat IgG. Protein G binds, primarily, antibodies in the Fc region, more specifically at the interface between CH2 and CH3 domains, however, some binding to the Fab region can occur. Protein A is expressed in *Staphylococcus aureus* and has five IgG binding domains. Unlike protein G, protein A, exhibits a narrower antibody binding range within the IgG class, since it does not bind human IgG3. However, it demonstrates affinity towards other immunoglobulin classes IgA, IgM, IgE, but its highest affinity is still exhibited towards IgG [150]. Finally, while protein G shows overall higher affinity to IgG, protein A usually is less expensive and, as a general rule, it is more often used in immunosensing applications [150]. In antibody immobilization procedures by using intermediate proteins such as protein A and G, antibodies are properly immobilized, but usually suffers from low surface density of immobilized antibodies. This method may also suffer from instability of the antibody–intermediate protein interaction [149].

Finally, several immunoassays have employed 3D surface structures with the aim to provide a more biocompatible environment and a high antibody loading capacity. As in the case of immobilization in a flat surface, antibodies can be simply adsorbed, diffused, or covalently linked to these matrix systems, however with the same constrains observed with the flat surface, but reaching a high capacity. Magnetic micro-beads (MBs) functionalized with a binding protein, like protein A or G, can be used as one of these 3D matrices with better results. Not only, the orientation of the immobilized antibodies is favorable due to MBs functionalization with a binding protein but the use of MBs will improve the affinity interaction thanks to a faster assay kinetics of the dispersed beads. Also, the surface area is higher than in a flat solid phase, and washing and separation efficiencies are enhanced due to the use of an external well-controlled magnetic field[150,152]. Furthermore, this type of 3D structures allow the analysis of complex samples without any pre-enrichment or purification steps [150,152].

Providing information of Ab orientation on the surface is, as previously reported, of fundamental interest. With this aim, many techniques have been used to elucidate the presence and binding function of immobilized antibodies. Different methods can be used to this end, namely: the Fourier transform infrared reflection (FTIR), the fluorescence microscopy, the atomic force microscopy (AFM), small-angle X-ray scattering (SAXs), the time-of-flight secondary ion mass spectrometry (ToF- SIMS), the dual polarization interferometry (DPI) and the neutron reflectometry (NR).

FTIR characterize the presence of specific chemical groups used for immobilization and various fluorescence microscopies help to visualize efficient binding of analyte to Ab-functionalized surfaces. These techniques are predominately used to roughly confirm an effective antibody immobilization [151].

AFM scans surfaces at nano-scale. 3-D structures of biomaterials such as proteins can be visualized, resulting in a topographical surface map. Through this technique, the surface coverage degree, layer thickness and proteins shape can be deduced [151].

SAXs gives us an enhanced insight into assembly and unfolding processes of macromolecules, a protein solution is exposed to x-rays, and the scattered intensity is recorded as a function of the scattering angle 2θ or the scattering vector *Q*. (Te x-ray pattern is determined by differences in the electron densities, which after analysis takes us to the protein three-dimensional structure [153].

ToF-SIMS allow to obtain information about the structure of the surface by providing biophysical evidence about the molecular structure. The resulting positively or negatively charged ions are analyzed, yielding a fingerprint of the proteins. As the sampling depth is very shallow, these data can be used to interpret the orientation of immobilized proteins [151].

DPI is an optical wave-guide-based analytical technique, which can be used to obtain information on molecular dimensions (layer thickness), packing (layer refractive index, density) and stoichiometry (mass). The measured layer thickness can provide information about antibodies arrangement on the surface when combined with known dimensions of the molecule[151].

NR is a neutron diffraction technique that determine the thickness and composition of molecular layers on surfaces. The technique involves directing a beam of neutrons onto a flat surface, and then the intensity of the reflected radiation is measured as a function of angle or neutron wavelength. By comparing the layer thickness with the molecular dimensions of antibodies it is possible to differentiate between flat-on, side-on and head-on/tail-on orientations (figure I.20) [151].

I.3.5.2. Antibody labels

Although the antigen–antibody reaction can cause changes in the electrochemical signal, the change is relatively small. In this way, for the successful development of an electrochemical immunosensor, signal amplification and noise reduction are crucial. To solve this limitation, high-affinity antibodies and appropriate labels are usually employed for the amplification of electrochemical signal. Antibody labels mainly contain bioactive enzymes, and electroactive materials, such as nanostructures, quantum dots (QD), and metal ions [105]. However it is important to clarify that there is no ideal label, and each of them has its own advantages and disadvantages [154].

Enzymatic labels are widely used because of the easiness with which they can be found from commercial sources and their extended application in ELISA (Section I.2.3.3.). These type of labels should catalyze the production of a specie that is electroactive, in order to be easily measured through electrochemical techniques [147]. Although large signal amplification can be achieved by enzyme labeling, the activity of the conjugate must be periodically controlled owing to the inherent poor stability of enzymes [104,155]. The use of *Horseradish peroxidase* (HRP), as an *Alkaline phosphatase* (AP) that conjugate antibodies, has been the preferred label for electrochemical immunosensors [147].

An alternative to enzyme labels are inorganic signal amplification tags, such as gold nanoparticles or quantum dots, which have demonstrated interesting results in terms of sensitivity, analysis time and correlation with standard methods [147]. Noble metal nanoparticles (NMNPs) possess exceeding advantages over other nanomaterials including stability, conductivity and biocompatibility. As previously mention (section I.3.4.1) when used on sensor surface, NMNPs conductivity facilitates the electron transfer between biological elements and electrode surface, and increase the surface area, allowing the stable immobilization of biomolecules, with their bioactivity maintained. Moreover, their stability and biocompatibility make them easy to conjugate with multiple species of biomolecules [156]. Other semiconductor nanostructures, such as carbon tubes and quantum dots (section I.3.4.1), have also been directly used as electroactive labels to amplify the signal in the electrochemical detection of proteins [105]. Nowadays, most commercially available immunoassays are based on the catalysis of the enzyme-labeled secondary antibodies [105]. Despite the fact that the electrochemical techniques allow labeling by inorganic tags, their sensitivities remain insufficient when compared with enzymatic labels [154].

I.3.6. Biosensors reported in the literature for HCMV detection

Few reports can be found in literature associated with HCMV new detection methods. Two of the proposed techniques are related with electrochemical recognition of amplified HCMV DNA sequences[157,158], using miniaturized sensing devices. Azek *et al.*[157] proposed, based on disposable screen-printed carbon electrodes (SPCEs), a biosensor for the detection of amplified HCMV DNA. Target DNA was adsorbed and hybridized with a biotinylated DNA probe and the formed hybrids were determined with streptavidin conjugated to *horseradish peroxidase*. Although large signal amplification has been achieved, the activity of the conjugate must be periodically controlled owing to enzyme stability. To overcome this limitation, Authier *et al.*[158] explored a strategy based on colloidal gold nanoparticle labels, for the sensitive quantification of an amplified 406-base pair human HCMV DNA sequence. Nevertheless, both described methods are used in conjunction with PCR, being costly and useless as point-of-care [157,158].

A piezoelectric sensor was also described by Susmel *et al.*[159] to detect HCMV glycoprotein; antibodies were immobilized on a gold electrode. Although the technique does not rely on amplified DNA, requires the use of expensive instrumentation.

Additionally, an immunofluorescence based detection device was developed by Wacogne *et al.*[160], here the biological sample is applied onto a gold surface coated with HCMV specific antibodies and if present in biological samples, HCMV is trapped onto the surface. Using

a fluorescent probe, positive and negative assays could be discriminated by fluorescence intensity. The main drawback of this device is the low sensitivity that compromises its applicability to samples with low viral loads.

Recently, an immunoassay for HCMV pp65 antigen detection using HRP and Pt-Pd nanoparticles functionalized single-walled carbon nanohorns was proposed [161]. The approach allowed rapid HCMV detection, however, the use of glassy carbon electrodes is not a practical alternative for a point-of-care method.

I.4. Aims

I.4.1. Aim of the thesis

The primary aim of this research is the development of an immunosensor model prototype that can be ~~developed~~established into a simple, low-cost, reliable and selective antibody-antigen interaction based sensor, for the detection of human cytomegalovirus in urine samples.

The research activity is focused on the construction of miniaturized disposable sensors. These devices will be used in a sandwich-type immunoassay by using antibodies against HCMV glycoprotein (gB), the major antigen on the envelope of HCMV.

Alongside with the primary aim are the specific goals, related with the different stages involved in the development of an immunosensor. These involve:

1. Selection of the general biosensor format:
   - Spectrophotometric detection vs electrochemical detection.
   - Screen printed carbon electrodes modified with nanostructures vs bare electrodes.
2. Selection of the appropriate methods for antibody immobilization:
   - Adsorption vs immobilization on magnetic particles.
3. Selection of the appropriate methods for secondary antibody labelling:
   Metallic nanoparticles labels vs Enzymes labels.
4. Immunosensor optimization:
   - Optimization of antibody concentration.
   - Reduction of unspecific adsorption (Blocking agents selection).
   - Optimization of the incubation conditions.
5. Characterization of the developed immunosensor:
   - Evaluation of the linear calibration range.
   - Evaluation of the detection limit.
   - Evaluation of immunosensor reproducibility.
   - Interference and selectivity studies.
6. Immunosensor validation by its application to human samples infected with human cytomegalovirus.

Different immuno~~s~~sensor configurations are tried, two of them use electrochemical detection coupled to the screen-printed microfabrication technology, that allows their application *in situ* and as point of care devices. The third configuration is based on spectrophotometric detection and on the assembly of a magnetic particle-based enzyme immunoassays (mpEIA), more appropriate when higher productivity is desired in the measurement of a large number of biological samples.

Bibliography

1. Revello, M. G. & Gerna, G. Diagnosis and Management of Human Cytomegalovirus Infection in the Mother, Fetus, and Newborn Infant. *Clin. Microbiol. Rev.* **15,** 680–715 (2002).
2. Sanchez, V., Greis, K. D., Sztul, E. & Britt, W. J. Accumulation of Virion Tegument and Envelope Proteins in a Stable Cytoplasmic Compartment during Human Cytomegalovirus Replication: Characterization of a Potential Site of Virus Assembly Accumulation of Virion Tegument and Envelope Proteins in a Stable C. *J. Virol.* **74,** 975–986 (2000).
3. Yen-Lieberman, B. Diagnosis of Human Cytomegalovirus Disease. *Clin. Microbiol. Newsl.* **22,** 105–109 (2000).
4. Boeckh, M. & Geballe, A. P. Science in medicine Cytomegalovirus : pathogen, paradigm, and puzzle. **121,** 1673–1680 (2011).
5. Landolfo, S., Gariglio, M., Gribaudo, G. & Lembo, D. The human cytomegalovirus. *Pharmacol. Ther.* **98,** 269–297 (2003).
6. Cannon, M. J., Schmid, D. S. & Hyde, T. B. Review of cytomegalovirus seroprevalence and demographic characteristics associated with. *Rev. Med.* **20,** 202–213 (2010).
7. *Human Cytomegalovirus*. *Human Cytomegalovirus* (Springer Berlin Heidelberg, 2008).
8. Pires, F. *et al.* Disposable immunosensor for human cytomegalovirus glycoprotein B detection. *Talanta* **136,** 42–46 (2015).
9. Macagno, A. *et al.* Isolation of human monoclonal antibodies that potently neutralize human cytomegalovirus infection by targeting different epitopes on the gH/gL/UL128-131A complex. *J. Virol.* **84,** 1005–1013 (2010).
10. Speckner, A., Glykofrydes, D., Ohlin, M. & Mach, M. Antigenic domain 1 of human cytomegalovirus glycoprotein B induces a multitude of different antibodies which, when combined, results in incomplete virus neutralization. *J. Gen. Virol.* **80,** 2183–2191 (1999).
11. Butcher, S. J., Aitken, J., Mitchell, J., Gowen, B. & Dargan, D. J. Structure of the human cytomegalovirus B capsid by electron cryomicroscopy and image reconstruction. *J. Struct. Biol.* **124,** 70–76 (1998).
12. Arvin, A. M. & Gershon, A. A. Varicella-Zoster Virus. *Clin. Microbiol. Rev.* **9,** 361–381 (1996).
13. Jenson, H. B. Epstein-Barr Virus. *Pediatr. Rev.* **32,** 375–384 (2011).
14. Levy, J. A. Three new human herpesviruses (HHV6, 7, and 8). *Lancet* **349,** 558–563 (1997).
15. Chentoufi, A. A. & Benmohamed, L. Mucosal herpes immunity and immunopathology to ocular and genital herpes simplex virus infections. *Clin. Dev. Immunol.* **2012,** 1–22 (2012).

16. Stevens, J. G. Human herpesviruses: a consideration of the latent state. *Microbiol. Rev.* **53,** 318–332 (1989).
17. Christensen, T. Human Herpesviruses in MS. *Int. MS J.* **14,** 41–47 (2007).
18. Grangeot-Keros, L. *et al.* Value of cytomegalovirus (CMV) IgG avidity test for the diagnosis of CMV infection in pregnant women. *J. Infect. Dis.* **175,** 944–946 (1997).
19. Mocarski, E. S., Shenk, T., Griffiths, P. & Pass, R. F. Cytomegalovirus. in *Fields Virology - Volume 2* (eds. Knipe, D. M. et al.) 1960–2014 (Lippincott Williams & Wilkins, a Wolters Kluwer business, 2013).
20. Plafker, S. M. & Gibson, W. Cytomegalovirus Assembly Protein Precursor and Proteinase Precursor Contain Two Nuclear Localization Signals That Mediate Their Own Nuclear Translocation and That of the Major Capsid Protein. *J. Virol.* **72,** 7722–7732 (1998).
21. Zini, N. *et al.* The Novel Structural Protein of Human Cytomegalovirus , pUL25 , Is Localized in the Viral Tegument. *J. Virol.* **73,** 6073–6075 (1999).
22. Spaete, R. R., Gehrz, R. C. & Landini, M. P. Human cytomegalovirus structural proteins. *J. Gen. Virol.* **75,** 3287–3308 (1994).
23. Britt, W. J. & Mach, M. Human cytomegalovirus glycoproteins. *Intervirology* **39,** 401–412 (1996).
24. Wille, P. T., Knoche, A. J., Nelson, J. A., Jarvis, M. A. & Johnson, D. C. A Human Cytomegalovirus gO-Null Mutant Fails To Incorporate gH / gL into the Virion Envelope and Is Unable To Enter Fibroblasts and Epithelial and Endothelial Cells. **84,** 2585–2596 (2010).
25. Theiler, R. N. & Compton, T. Characterization of the Signal Peptide Processing and Membrane Association of Human Cytomegalovirus Glycoprotein O. *J. Biol. Chem.* **276,** 39226–39231 (2001).
26. Mach, M., Kropff, B., Dal Monte, P. & Britt, W. Complex Formation by Human Cytomegalovirus Glycoproteins M (gpUL100) and N (gpUL73). *J. Virol.* **74,** 11881–11892 (2000).
27. Gretch, D. R., Kari, B., Rasmussen, L., Gehrz, R. C. & Stinski, M. F. Identification and Characterization of Three Distinct Families of Glycoprotein Complexes in the Envelopes of Human Cytomegalovirus. *J. Virol.* **62,** 875–881 (1988).
28. Kari, B., Li, W., Cooper, J., Goertz, R. & Radeke, B. The human cytomegalovirus UL100 gene encodes the gC-II glycoproteins recognized by Group 2 monoclonal antibodies. *J. Gen. Virol.* **75,** 3081–3086 (2018).
29. Griffiths, P., Baraniak, I. & Reeves, M. The pathogenesis of human cytomegalovirus. *J. Pathol.* **235,** 288–297 (2015).
30. Speckner, A., Kropff, B., Knör, S. & Mach, M. The antigenic domain 1 of human cytomegalovirus glycoprotein B contains an intramolecular disulphide bond. *J. Gen. Virol.* **81,** 2659–2663 (2000).
31. Crough, T. & Khanna, R. Immunobiology of Human Cytomegalovirus: from Bench to Bedside. *Clin. Microbiol. Rev.* **22,** 76–98 (2009).

32. Mocarski Jr, E. S. Comparative analysis of herpesvirus-common proteins. in *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis* (eds. Arvin, A. et al.) (Cambridge Universyty Press, 2007).
33. Sinclar, J. & Sissons, P. Latent and Persistent infections of monocytes and macrophages. *Intervirology* **39,** 293–301 (1996).
34. Stinski, M. F. & Isomura, H. Role of the cytomegalovirus major immediate early enhancer in acute infection and reactivation from latency. *Med. Microbiol. Immunol.* **197,** 223–231 (2008).
35. Mattes, F. M., McLaughlin, J. E., Emery, V. C., Clark, D. A. & Griffiths, P. D. Histopathological detection of owl's eye inclusions is still specific for cytomegalovirus in the era of human herpesviruses 6 and 7. *J. Clin. Pathol.* **53,** 612–614 (2000).
36. Bissinger, A. L., Sinzger, C., Kaiserling, E. & Jahn, G. Human cytomegalovirus as a direct pathogen: Correlation of multiorgan involvement and cell distribution with clinical and pathological findings in a case of congenital inclusion disease. *J. Med. Virol.* **67,** 200–206 (2002).
37. Sinzger, C. & Jahn, G. Human Cytomegalovirus Cell Tropism and Pathogenesis. *Interviology* **39,** 302–319 (1996).
38. Streblow, D. N. & Nelson, J. A. Models of HCMV latency and reactivation. *Trends Microbiol.* **11,** 293–295 (2003).
39. Carroll, M. C., Sally, E., Ravetch, J., Kelsoe, D. & Fearon, D. The humoral immune response. in *Immunobiology: The immune system in health and disease* (eds. Janeway, Ch. A., Travers, P., Walport, M. & Shlomchik, M.) (Garland Publishing, 2018).
40. Paulnock, D., Springer, T., Jenking, M. K., Tschopp, J. & Steimman, R. T-Cell mediated-immunity. in *Immunobiology: The immune system in health and disease* (eds. Janeway, Ch. A., Travers, P., Walport, M. & Shlomchik, M.) (Garland Publishing, 2018).
41. Slifka, M. K., Antia, R., Whitmire, J. K. & Ahmed, R. Humoral immunity due to long-lived plasma cells. *Immunity* **8,** 363–372 (1998).
42. Kumar, D. *et al.* Cell-mediated immunity to predict cytomegalovirus disease in high-risk solid organ transplant recipients. *Am. J. Transplant.* **9,** 1214–1222 (2009).
43. Rosenberg, J. & Huang, J. CD8+T cells and NK cells: parallel and complementary soldiers of immunotherapy. *Curr. Opin. Chem. Eng.* **19,** 9–20 (2018).
44. Iwasaki, A. & Medzhitov, R. Innate Responses to Viral Infections. in *Field Virology - Volume 1* (eds. Knipe, D. M. et al.) 189–213 (Lippincott Williams & Wilkins, a Wolters Kluwer business, 2013).
45. Kärre, K. NK cells, MHC class I molecules and the missing self. *Scand. J. Immunol.* **55,** 221–228 (2002).
46. Naing, Z. W. *et al.* Congenital cytomegalovirus infection in pregnancy: A review of prevalence, clinical features, diagnosis and prevention. *Aust. New Zeal. J. Obstet. Gynaecol.* **56,** 9–18 (2016).
47. Enders, G., Daiminger, A., Bäder, U., Exler, S. & Enders, M. Intrauterine transmission

and clinical outcome of 248 pregnancies with primary cytomegalovirus infection in relation to gestational age. *J. Clin. Virol.* **52,** 244–246 (2011).

48. Kenneson, A. & Cannon, M. J. Review and meta-analysis of the epidemiology of congenital cytomegalovirus (CMV) infection. *Rev. Med. Virol.* **17,** 253–276 (2007).
49. Duff, P. *Diagnosis and Management of CMV Infection in Pregnancy*. *Perinatology* **1,** (2010).
50. Demmler-Harrison, G. J. Congenital cytomegalovirus: Public health action towards awareness, prevention, and treatment. *J. Clin. Virol.* **46S,** 1–5 (2009).
51. Leung, A. K. C., Sauve, R. S. & Davies, H. D. Congenital cytomegalovirus infection. *J. Natl. Med. Assoc.* **95,** 213–218 (2003).
52. McVoy, M. A. Cytomegalovirus Vaccines. *Clin. Infect. Dis.* **57,** S196–S199 (2013).
53. Adler, S. P. *et al.* Immunity Induced by Primary Human Cytomegalovirus-Infection Protects against Secondary Infection among Women of Childbearing Age. *J. Infect. Dis.* **171,** 26–32 (1995).
54. Adler, S. P. *et al.* A Phase 1 Study of 4 Live, Recombinant Human Cytomegalovirus Towne/Toledo Chimera Vaccines in Cytomegalovirus-Seronegative Men. *J. Infect. Dis.* **214,** 1341–1348 (2016).
55. Zhang, C., Evans, A., Simpson, T., Andrews, W., Huang, M. L. Vaccine prevention of maternal cytomegalovirus infection. *N. Engl. J. Med.* **360,** 1191–1199 (2009).
56. Diamond, D. J., York, J., Sun, J. Y., Wright, C. L. & Forman, S. J. Development of a candidate HLA A*0201 restricted peptide-based vaccine against human cytomegalovirus infection. *Blood* **90,** 1751–1767 (1997).
57. Hillyer, C. D., Snydman, D. R. & Berkman, E. M. The risk of cytomegalovirus infection in solid organ and bone marrow transplant recipients: Transfusion of blood products. *Transfusion* **30,** 659–666 (1990).
58. Mandong, G., Yanqing, L., Hongxia, G., Xiaoqin, W. & Lifang, F. Electrochemical detection of short sequences related to the hepatitis B virus using MB on chitosan-modified CPE. *Bioelectrochemistry* **70,** 245–249 (2007).
59. Gallant, J. E., Moore, R. D., Richman, D. D., Keruly, J. & Chaisson, R. E. Incidence and Natural History of Cytomegaloviurs Disease in Patients with advanced human immunodeficiency virus disease treated with zidovudine. *J. Infect. Dis.* **166,** 1223–1227 (1992).
60. Ross, S. A., Novak, Z., Pati, S. & Boppana, S. B. Diagnosis of cytomegalovirus infection. *Infect. Disord. Drug Targets* **11,** 466–474 (2011).
61. Pereira, L., Maidji, E., McDonagh, S., Genbacev, O. & Fisher, S. Human cytomegalovirus transmission from the uterus to the placenta correlates with the presence of pathogenic bacteria and maternal immunity. *J. Virol.* **77,** 13301–13314 (2003).
62. Boppana, S. B., Fowler, K. B., Britt, W. J., Stagno, S. & Pass, R. F. Symptomatic Congenital Cytomegalovirus Infection in Infants Born to Mothers With Preexisting Immunity to Cytomegalovirus. *Pediatrics* **104,** 55–60 (1999).

63. Stagno, S. *et al*. Primary Cytomegalovirus Infection in Pregnancy. ncidence, transmission to fetus, and clinical outcome. *JAMA* **256,** 1904–1908 (1986).

64. Gindes, L., Teperberg-Oikawa, M., Sherman, D., Pardo, J. & Rahav, G. Congenital cytomegalovirus infection following primary maternal infection in the third trimester. *BJOG An Int. J. Obstet. Gynaecol.* **115,** 830–835 (2008).

65. Bodéus, M., Feyder, S. & Goubau, P. Avidity of IgG antibodies distinguishes primary from non-primary cytomegalovirus infection in pregnant women. *Clin. Diagn. Virol.* **9,** 9–16 (1998).

66. Boppana, S. B., Rivera, L. B., Fowler, K. B., Mach, M. & Britt, W. J. Intrauterine transmission of cytomegalovirus to infants of women with preconceptional immunity. *N. Engl. J. Med.* **344,** 1366–1371 (2001).

67. Baalawi, F., Robertson, P. & Rawlinson, W. D. Detection of human cytomegalovirus IgG antibody using automated immunoassay with recombinant antigens gives uniform results to established assays using whole virus antigens. *Pathology* **42,** 578–580 (2010).

68. Rasmussen, L. *et al*. Virus-specific IgG and IgM antibodies in normal and immunocompromised subjects infected with cytomegalovirus. *J. Infect. Dis.* **145,** 191–9 (1982).

69. Blackburn, N. K., Besselaar, T. G., Schoub, B. D. & O'Connell, K. F. Differentiation of primary cytomegalovirus infection from reactivation using the urea denaturation test for measuring antibody avidity. *J. Med. Virol.* **33,** 6–9 (1991).

70. Sun, H. *et al*. Detection of cytomegalovirus antibodies using a biosensor based on imaging ellipsometry. *PLoS One* **10,** 1–12 (2015).

71. Schmitz, H. & Haas, R. Determination of Different Cytomegalovirus Immunoglobulins (IgG , IgA , IgM) by Immnnofluorescence By. *Arch. Virol.* **37,** 131–140 (1972).

72. Strand, O. A. & Hoddevik, G. M. The Diagnostic Significance of Specific Serum IgA Detection in Cytomegalovirus Infections By. *Arch. Virol.* **82,** 173–180 (1984).

73. Mazzulli, T., Wood, S., Chua, R. & Walmsley, S. Evaluation of the digene hybrid capture system for detection and quantitation of human cytomegalovirus viremia in human immunodeficiency virus-infected patients. *J. Clin. Microbiol.* **34,** 2959–2962 (1996).

74. Collins, M. L. *et al*. A branched DNA signal amplification assay for quantification of nucleic acid targets below 100 molecules/ml. *Nucleic Acids Res.* **25,** 2979–2984 (1997).

75. Ghindilis, A. L., Atanasov, P., Wilkins, M. & Wilkins, E. Immunosensors: electrochemical sensing and other engineering approaches. *Biosens. Bioelectron.* **13,** 113–131 (1998).

76. Voller, A., Bartlett, A. & Bidwell, D. E. Enzyme immunoassays with special reference to ELISA techniques. *J. Clin. Pathol.* **31,** 507–520 (1978).

77. Holford, T. R. J., Davis, F. & Higson, S. P. J. Recent trends in antibody based sensors. *Biosens. Bioelectron.* **34,** 12–24 (2012).

78. Voller, A., Bidwell, D. E. & Bartlett, A. Enzyme immunoassays in diagnostic medicine. Theory and practice. *Bull. World Health Organ.* **53,** 55–65 (1976).

79. Bourgeois, M. A. & Oak, J. L. Laboratory Diagnosis of Viral Infections. in *Equine*

Infectious Diseases (eds. C. Sellon, D. & Long, M. T.) 132–140 (Elsevier, 2014).

80. Goding, J. W. Immunofluorescence. in *Monoclonal Antibodies: principles and practice* 352–399 (Academic Press Limited, 1996).
81. Luppa; Peter B.; Sokoll, Lori; Chan, D. W. Immunosensors- Principles and applications to clinical chemistry. *Clin. Chim. Acta* **314,** 1–26 (2001).
82. Davies, D. R. & Cohen, G. H. Interactions of protein antigens with antibodies. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 7–12 (1996).
83. Peakman, M. & Vergani, D. Acquired immunity: antigen receptors. in *Basic and Clinical immunology* (eds. Peakman, M. & Vergani, D.) (Churcill Livingstone Elsevier, 2009).
84. Branden, B. C. & Poljak, R. J. Structural features of the reactions between antibodies and protein antigens. *FASEB J.* **9,** 9–16 (1995).
85. Sela-Culang, I., Kunik, V. & Ofran, Y. The structural basis of antibody-antigen recognition. *Front. Immunol.* **4,** 1–13 (2013).
86. Gopinath, S. C. B., Tang, T. H., Citartan, M., Chen, Y. & Lakshmipriya, T. Current aspects in immunosensors. *Biosens. Bioelectron.* **57,** 292–302 (2014).
87. Cohen, S. & Milstein, C. Structure and biological properties of immunoglobulins. *Adv. Immunol.* **7,** 1–89 (1967).
88. De La Escosura-Muñiz, A., Parolo, C. & Merkoçi, A. Immunosensing using nanoparticles. *Mater. Today* **13,** 24–34 (2010).
89. Vidarsson, G., Dekkers, G. & Rispens, T. IgG subclasses and allotypes: From structure to effector functions. *Front. Immunol.* **5,** 1–17 (2014).
90. Gutzeit, C., Chen, K. & Cerutti, A. The enigmatic function of IgD: some answers at last. *Eur. J. Immunol.* 1–13 (2018). doi:10.1002/eji.201646547
91. Donahue, A. C. & Albitar, M. Antibodies in Biosensing. in *Recognition Receptors in Biosensors* (ed. Zourob, M.) 221–248 (Springer, 2010).
92. Lipman, N. S., Jackson, L. R., Trudel, L. J. & Weis-Garcia, F. Monoclonal Versus Polyclonal Antibodies: Distinguishing Characteristics, Applications, and Information Resources. *ILAR J.* **46,** 258–268 (2005).
93. Filpula, D. Antibody engineering and modification technologies. *Biomol. Eng.* **24,** 201–215 (2007).
94. Holliger, P., Prospero, T. & Winter, G. 'Diabodies': small bivalent and bispecific antibody fragments. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 6444–6448 (1993).
95. Nelson, A. L. Antibody fragments: hope and hype. *Landes Biosci.* **2,** 77–83 (2010).
96. Borrebaeck, C. A. K. Antibody in diagnostics - from immunassays to protein chips. *Immunol. Today* **21,** 379–382 (2000).
97. Voller, A. & O'Neill, P. Immunofluorescence method suitable for large-scale application to malaria. *Bull. World Health Organ.* **45,** 524–529 (1971).
98. Rosendal, S. & Black, F. T. Direct and Indirect Immunofluorescence of Unfixed and Fixed Mycoplasma Colonies. *Acta Pathol. Microbiol. Scand. Sect. B Microbiol. Immunol.* **80,** 615–622 (1972).

99. Akikusu, J. & Choo, S. Laboratory Investigations. in *Textbook of pediatric rheumatology* (eds. Petty, R. E., Laxer, R., Lindsley, C. & Weddeburn, L.) 117–128.e6 (Elsevier Inc., 2016).
100. Burrel, C. J., Howard, C. R. & Murphy, F. A. Laboratory Diagnosis of virus Diseases. in *Fenner and White's Medical VIrology* 135–154 (Elsevier Inc., 2017).
101. Vidal, J. C. *et al*. Rapid determination of recent cocaine use with magnetic particles-based enzyme immunoassays in serum, saliva, and urine fluids. *J. Pharm. Biomed. Anal.* **125,** 54–61 (2016).
102. Butler, J. E. Enzyme-Linked Immunosorbent Assay. *J. Immunoassay* **21,** 165–209 (2000).
103. Cox, K. L. *et al*. Immunoassays Methods. in *Assay Guidance Manual* (eds. Sittampalam, G. S. et al.) (2012).
104. Warsinke, A., Benkert, A. & Scheller, F. W. Electrochemical immunoassays. *J. Anal. Chem.* **366,** 622–634 (2000).
105. Pei, X. *et al*. Sandwich-type immunosensors and immunoassays exploiting nanostructure labels: A review. *Anal. Chim. Acta* **758,** 1–18 (2013).
106. Morrison, D. W. G., Dokmeci, M. R., Demirci, U. & Khademhosseini, A. Clinical Applications of Micro- and Nanoscale Biosensors. in *Biomedical Nanostructures* (eds. Gonsalves, K. E., Halberstadt, C. R., Laurencin, C. T. & Nair, L. S.) 439–454 (2008).
107. Caygill, R. L., Blair, G. E. & Millner, P. a. A review on viral biosensors to detect human pathogens. *Anal. Chim. Acta* **681,** 8–15 (2010).
108. Clark, L. C. & Lyons, C. Electrode Systems for Continuous Monitoring in Cardiovascular Surgery. *Ann. N. Y. Acad. Sci.* **102,** 29–45 (1962).
109. Perumal, V. & Hashim, U. Advances in biosensors: Principle, architecture and applications. *J. Appl. Biomed.* **12,** 1–15 (2014).
110. Grieshaber, D., MacKenzie, R., Vörös, J. & Remhult, E. Electrochemical Biosensors - Sensor Principles and Architectures. *Sensors* **8,** 1400–1458 (2008).
111. Thévenot, D. R., Toth, K., Durst, R. A. & Wilson, G. S. Technical report Electrochemical biosensors : recommended definitions and classification. *Biosens. Bioelectron.* **16,** 121–131 (2001).
112. Burcu Bahadir, E. & Kemal Sezgintürk, M. Applications of electrochemical immunosensors for early clinical diagnostics. *Talanta* **132,** 162–174 (2015).
113. Yadav, R., Dwivedi, S., Kumar, S. & Chaudhury, A. Trends and perspectives of biosensors for food and environmental virology. *Food Environ. Virol.* **2,** 53–63 (2010).
114. Monošík, R., Streďanský, M. & Šturdík, E. Biosensors - classification, characterization and new trends. *Acta Chim. Slovaca* **5,** 109–120 (2012).
115. Patel, P. . (Bio)sensors for measurement of analytes implicated in food safety: a review. *TrAC Trends Anal. Chem.* **21,** 96–115 (2002).
116. Mello, L. D. & Kubota, L. T. Review of the use of biosensors as analytical tools in the food and drink industries. *Food Chem.* **77,** 237–256 (2002).
117. Frew, J. E. & Hill, H. a. Electrochemical biosensors. *Anal. Chem.* **59,** 933A–944A (1987).

118. Leca-Bouvier, B. D. & Blum, L. J. *Enzyme for Biosensing Applicationseca-bouvier, D.* (2010). doi:10.1007/978-1-4419-0919-0
119. Campàs, M., Carpentier, R. & Rouillon, R. Plant tissue-and photosynthesis-based biosensors. *Biotechnol. Adv.* **26,** 370–378 (2008).
120. D'Orazio, P. Biosensors in clinical chemistry. *Clin. Chim. Acta* **334,** 41–69 (2003).
121. Wang, J. Electrochemical nucleic acid Biosensors. *Anal. Chim. Acta* **469,** 63–71 (2002).
122. Liu, Y., Tuleouva, N., Ramanculov, E. & Revzin, A. Aptamer-based electrochemical biosensor for interferon gamma detection. *Anal. Chem.* **82,** 8131–8136 (2010).
123. Rodriguez, M. C., Kawde, A. N. & Wang, J. Aptamer biosensor for label-free impedance spectroscopy detection of proteins based on recognition-induced switching of the surface charge. *Chem. Commun.* 4267–4269 (2005). doi:10.1039/b506571b
124. Yan, F., Wang, F. & Chen, Z. Aptamer-based electrochemical biosensor for label-free voltammetric detection of thrombin and adenosine. *Sensors Actuators, B Chem.* **160,** 1380–1385 (2011).
125. Lazcka, O., Campo, F. & Muñoz, F. Pathogen detection: A prespective of traditional methods and biosensors. *Biosens. Bioelectron.* **22,** 1205–1217 (2007).
126. Kong, F. & Hu, Y. F. Biomolecule immobilization techniques for bioactive paper fabrication. *Anal. Bioanal. Chem.* **403,** 7–13 (2012).
127. Scouten, W. H., Luong, J. H. T. & Brown, R. S. Enzyme or protein immobilization techniques for applications in biosensor design. *Trends Biotechnol.* **13,** 178–185 (1995).
128. Albareda-Sirvent, M., Merkoçi, A. & Alegret, S. Configurations used in the design of screen-printed enzymatic biosensors . A review. *Sensors Actuators B* **69,** 153–163 (2000).
129. Luong, J. H. T., Male, K. B. & Glennon, J. D. Biosensor technology: technology push versus market pull. *Biotechnol. Adv.* **26,** 492–500 (2008).
130. Ronkainen, N. J., Halsall, H. B. & Heineman, W. R. Electrochemical biosensors. *Chem. Soc. Rev.* **39,** 1747–1763 (2010).
131. Alonso-Lomillo, M. A., Domínguez-Renedo, O. & Arcos-Martínez, M. J. Screen-printed biosensors in microbiology:a review. *Talanta* **82,** 1629–1636 (2010).
132. Renedo, O. D., Alonso-Lomillo, M. A. & Martínez, M. J. A. Recent developments in the field of screen-printed electrodes and their related applications. *Talanta* **73,** 202–219 (2007).
133. Taleat, Z., Khoshroo, A. & Mazloum-Ardakani, M. Screen-printed electrodes for biosensing: A review (2008-2013). *Microchim. Acta* **181,** 865–891 (2014).
134. Li, M., Li, Y. T., Li, D. W. & Long, Y. T. Recent developments and applications of screen-printed electrodes in environmental assays-A review. *Anal. Chim. Acta* **734,** 31–44 (2012).
135. Zhang, S., Wright, G. & Yang, Y. Materials and techniques for electrochemical biosensor design and construction. *Biosens. Bioelectron.* **15,** 273–282 (2000).
136. White, N. M. & Turner, J. D. Thick-film sensors: past, present and future. *Meas. Sci. Technol.* **8,** 1–20 (1997).

137. Wang, J. Nanomaterial-based electrochemical biosensors. *Analyst* **130,** 421–426 (2005).
138. Luo, Xiliang; Morrin, Aoife; Killard, Anthony J.; Smyth, M. R. Application of Nanoparticles in Electrochemical Sensors and Biosensors. *Electroanalysis* **18,** 319–326 (2006).
139. Welch, C. M. & Compton, R. G. The use of nanoparticles in electroanalysis: A review. *Anal. Bioanal. Chem.* **384,** 601–619 (2006).
140. Pingarrón, J. M., Yáñez-Sedeño, P. & González-Cortés, A. Gold nanoparticle-based electrochemical biosensors. *Electrochim. Acta* **53,** 5848–5866 (2008).
141. Tashkhourian, J., Nezhad, M. R. H., Khodavesi, J. & Javadi, S. Silver nanoparticles modified carbon nanotube paste electrode for simultaneous determination of dopamine and ascorbic acid. *J. Electroanal. Chem.* **633,** 85–91 (2009).
142. Rocha-Santos, T. A. P. Sensors and biosensors based on magnetic nanoparticles. *Trends Anal. Chem.* **62,** 28–36 (2014).
143. Zhou, H. *et al.* The sandwich-type electrochemiluminescence immunosensor for α-fetoprotein based on enrichment by Fe3O4-Au magnetic nano probes and signal amplification by CdS-Au composite nanoparticles labeled anti-AFP. *Anal. Chim. Acta* **746,** 107–113 (2012).
144. Liu, G. & Lin, Y. Nanomaterial labels in electrochemical immunosensors and immunoassays. *Talanta* **74,** 308–317 (2007).
145. Sun, H., Wu, L., Wei, W. & Qu, X. Recent advances in graphene quantum dots for sensing. *Mater. Today* **16,** 433–442 (2013).
146. Hock, B. Antibodies for immunosensors. *Anal. Chim.* **347,** 177–186 (1997).
147. Ricci, Francesco; Adornetto, Gianluca; Palleschi, G. A review of experimental aspects of electrochemical immunosensors. *Electrochim. Acta* **84,** 74–83 (2012).
148. Jung, Y., Jeong, J. Y. & Chung, B. H. Recent advances in immobilization methods of antibodies on solid supports. *Analyst* **133,** 697–701 (2008).
149. Peluso, P. *et al.* Optimizing antibody immobilization strategies for the construction of protein microarrays. *Anal. Biochem.* **312,** 113–124 (2003).
150. Makaraviciute, A. & Ramanaviciene, A. Site-directed antibody immobilization techniques for immunosensors. *Biosens. Bioelectron.* **50,** 460–471 (2013).
151. Trilling, A. K., Beekwilder, J. & Zuilhof, H. Antibody orientation on biosensor surfaces: a minireview. *Analyst* **138,** 1619–1627 (2013).
152. Centi, S., Stoica, A. I., Laschi, S. & Mascini, M. Development of an electrochemical immunoassay based on the use of an eight-electrodes screen-printed array coupled with magnetic beads for the detection of antimicrobial sulfonamides in honey. *Electroanalysis* **22,** 1881–1888 (2010).
153. Horejs, C. *et al.* Surface layer protein characterization by small angle x-ray scattering and a fractal mean force concept: from protein structure to nanodisk assemblies. *J. Chem. Phys.* **133,** 1–8 (2010).
154. Dequaire, M., Degrand, C. & Limoges, B. An electrochemical metalloimmunoassay based

on a colloidal gold label. *Anal. Chem.* **72,** 5521–5528 (2000).

155. Zhu, C., Yang, G., Li, H., Du, D. & Lin, Y. Electrochemical sensors and biosensors based on nanomaterials and nanostructures. *Anal. Chem.* **87,** 230–249 (2015).
156. Wang, J. Electrochemical biosensing based on noble metal nanoparticles. *Microchim. Acta* **177,** 245–270 (2012).
157. Azek, F., Grossiord, C., Joannes, M., Limoges, B. & Brossier, P. Hybridization Assay at a Disposable Electrochemical Biosensor for the Attomole Detection of Amplified Human Cytomegalovirus DNA. *Anal. bBochemistry* **284,** 107–113 (2000).
158. Authier, L., Grossiord, C. & Brossier, P. Gold Nanoparticles-Based Quantitative Electrochemical Detection of Amplified Human Cytomegalovirus DNA Using Disposable Microband Electrodes. *Anal. Chem.* **73,** 4450–4456 (2001).
159. Susmel, S., O'Sullivan, C. & Guilbault, G. Human cytomegalovirus detection by a quartz crystal microbalance immunosensor. *Enzyme Microb. Technol.* **27,** 639–645 (2000).
160. Wacogne, B. *et al.* The MEDICALIP Project: Toward the screening of the cytomegalovirus. *IRBM* **32,** 66–68 (2011).
161. Huang, W. *et al.* Electrochemical Immunoassay for *Cytomegalovirus* Antigen Detection with Multiple Signal Amplification Using HRP and Pt-Pd Nanoparticles Functionalized Single-walled Carbon Nanohorns. *Electroanalysis* **28,** 1126–1133 (2016).
162. Bertok, T. *et al.* Label-free detection of glycoproteins by the lectin biosensor down to attomolar level using gold nanoparticles. *Talanta* **108,** 11–18 (2013).
163. Pires, F., Dias-Cabral, A. C., Arcos-Martínez, M. J., Vidal, J. C. & Castillo, J. R. A rapid magnetic particle-based enzyme immunoassay for human cytomegalovirus glycoprotein B quantification. *J. Pharm. Biomed. Anal.* **156,** 372–378 (2018).

CHAPTER II

Disposable immunosensor for human cytomegalovirus glycoprotein B detection

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Disposable immunosensor for human cytomegalovirus glycoprotein B detection

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Keywords

Immunosensor, Screen-printed Electrodes; Human Cytomegalovirus; Glycoprotein B; Anodic stripping analysis

II.1. Summary

Combining the advantages of the biosensor field with the problematic of detecting Human cytomegalovirus (HCMV) in human samples, it is proposed an inexpensive, simple and disposable electrochemical immunosensor for glycoprotein B (gB) detection in urine. gB has been chosen once is the dominant antigen of HCMV. The approach is based on a sandwich-type immunoassay, with the HCMV gB sandwiched between the Anti-HCMV antibody adsorbed onto the working electrode, and the Anti-HCMV labeled with gold nanoparticles. The gB detection was carried out through electrochemical stripping analysis of silver nanoparticles quantitatively deposited on the immunosensor through catalysis by the nanogold labels. The influence of different steps in the immunosensor construction, namely the silver deposition time, silver enhancer concentration, antibody concentration, BSA concentration and gB incubation time were examined and optimized. The method showed a linear dependence between gB concentration and corresponding anodic stripping peak current, resulting in detection limits of 3.3 ± 1.7 ng mL^{-1} for samples prepared in buffer and 3.2 ± 0.2 ng mL^{-1} for urine samples, suggesting that the biological matrix does not interfere with the immunosensor detection capability. Given its mode of preparation, by physical adsorption of the capture antibody in the working electrode, the immunosensor also exhibited an acceptable reproducibility, with a residual standard deviation of 13.5% for samples prepared in buffer and 11.2 % for urine samples, thereby presenting a promising development potential for clinical applications.

II.2. Introduction

Human Cytomegalovirus (HCMV) is the most usual name for human herpesvirus 5 [1,2], like all herpes viruses, HCMV undergoes latency and reactivation in the host cell, so once a person is infected, the virus is not eliminated from the body but persists to cause a low grade chronic infection or remains in latent stage allowing further transmission of the virus to new hosts [1,3]. Humans are the only known receptors for this virus and the transmission is facilitated by mucous contact. The reactivation of HCMV to a state of active replication with potential to induce disease and transmission to a new host can occur in a situation of cellular immunity suppression induced by a disease [2]. Thereby, in immunosuppressed individuals, as transplanted ones, persons infected by the Human immunodeficiency virus (HIV) and those with immature immune system, like fetuses and newborns, the infection can be severe or even fatal [1,2,4]. All of the usually used methods for HCMV detection require a long period of time to perform or are costly which is problematic to use as point of care. Thus, there is a need to develop a method which is fast, effective and inexpensive for the diagnosis of this virus.

In recent years, electrochemical biosensors were widely used to determine various substances with different properties and for continuous monitoring of biological processes [5,6].

Bioanalytical assays such as immunoassays (IAs), which use specific antigen-antibody complexation, are also very important in many fields [7]. Moreover, IAs with electrochemical detection can offer enhanced sensitivities and reduced instrumentation costs compared to their counterparts using other transducing elements [5]. Warsinke et al.[8] already showed electrochemical IAs as promising alternatives to existing immunochemical tests for the development of handheld devices which can be used for point of care measurements. Also, screen printed electrodes (SPE) contribute to develop miniaturized, easy to handle, reliable, versatile and inexpensive IAs devices, which produce results within a few minutes [5].

Accurate detection of HCMV glycoprotein B (gB) in body fluids, like urine and saliva, where viral loads are higher, through immunoassay methods shows promising applications in screening and diagnosis of infection by HCMV. During the HCMV acute infection, specific antibodies are generated against to a large number of structural and non-structural proteins, but just glycoproteins B and H induce antibodies capable to neutralize virus and to eliminate infected cells. Furthermore, gB is the dominant antigen existing in the envelope of HCMV and approximately 100% of infected individuals develop antibodies against this protein. Thus, HCMV gB can be viewed as a promising component to be used in the development of diagnostic tests for HCMV [2,9]. In this work, using the screen-printed technology, an electrochemical immunosensor is developed for HCMV gB detection.

Once antibodies are usually not electrochemically active within the desired potential range, redox-active compounds need to be applied as labels [8]. With great interest on metalloimmunoassay, is the silver enhancement catalyzed by gold nanoparticles (AuNPs), thereby once these nanoparticles have well-known catalytic properties on silver ions reduction [10]. Thus, the use of a sandwich type immunoassay with a secondary antibody labelled with AuNPs (ab-AuNPs), allows the quantitative precipitation of silver nanoparticles (AgNPs), thereby improving the sensitivity of immunosensor [11]. Subsequent quantification is made by dissolving the AgNPs and measuring its oxidation by anodic stripping voltammetry. The present work describes a sandwich type immunosensor for the detection of HCMV gB using the upper mentioned principle (figure II.1.). To the best of our knowledge this is the first time such detection method is used in the quantification of this HCMV envelope glycoprotein. Also, the developed biosensor was proved to be effective in the detection of this glycoprotein, when present in human urine samples.

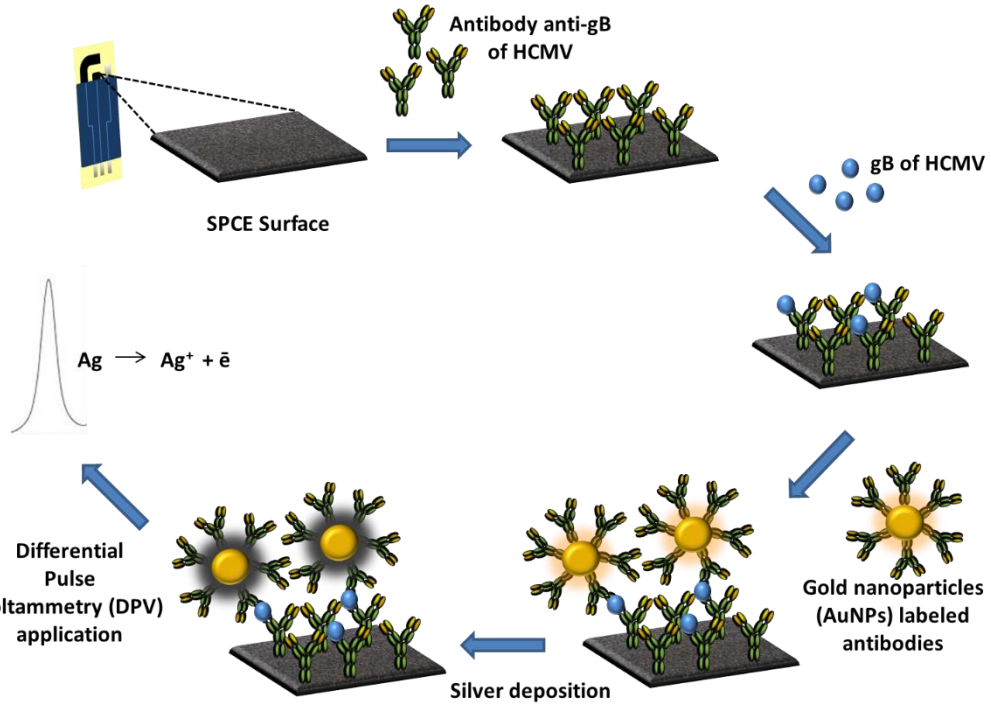

Figure II.1. Sandwich type immunosensor construction

II.3. Material and Methods

II.3.1. Reagents and apparatus

Cytomegalovirus glycoprotein B and Anti-Cytomegalovirus glycoprotein B antibody were purchased from Abcam, Cambridge, United Kingdom. Bovine serum albumin (BSA), chloroauric acid, Tris ((hydroxymethyl)aminomethane), nitric acid, potassium carbonate, trissodium citrate, potassium chloride, Tween-20 and silver enhancer solutions A and B were obtained from Sigma-Aldrich, Madrid, Spain. Urine samples were obtained from healthy volunteers who had given informed consent. All the reagents used were of analytical grade and Milli Q water (Millipore, Bedford, USA) was employed for preparing all solutions.

In the fabrication of screen-printed electrodes several inks were used, namely Electrodag PF-407 A (carbon ink), Electrodag 6037 SS (silver/silver chloride ink) and Electrodag 452 SS (dielectric ink) supplied by Achenson Colloiden (Scheemda, Netherlands). The SPEs, composed by carbon auxiliary and working electrodes and a Ag/AgCl pseudo-reference electrode, were produced on a DEK 248printing machine (DEK, Weymouth, United Kingdom), using polyester screens with appropriate stencil designs mounted at 45º to the printer stroke.

Electrochemical measurements were made using an Autolab PGSTAT128N electrochemical system with the General Purpose Electrochemical System (GPES) software version 4.9 (Echo

Chemie, Utrecht, Netherlands). The solutions pH was measured with a Hanna instruments HI 221 pH meter (USA).

II.3.2 Preparation of Au NP-labeled antibodies

For the preparation of colloidal AuNps, 100 mL of 0.01% $HAuCl_4$ solution were boiled with vigorous stirring, and 2.5 mL of 1% trisodium citrate solution was quickly added to the boiling solution. It is expected that the solution becomes red, indicating the formation of AuNps. After this, the solution was left stirring in order to cool down [12].

The Au-labeled antibody was obtained by adding 10 µL of a 1 mg mL^{-1} antibody solution to a cold colloidal gold suspension (1 mL) adjusted to pH 9.0, with 0.1 M K_2CO_3 solution. Next the solution was stirred at room temperature for 60 minutes. Then, the conjugate was centrifuged at 4800 rpm for 30 minutes, twice. Finally, the soft sediment was suspended in 50 mM pH 7.2 Tris-HNO_3 solution (containing 1% BSA).The solution was stored at 4ºC [12,13].

II.3.3. Immunosensor preparation and measurement procedure

The immunosensor was constructed by adsorption immobilization of corresponding capture antibodies on the screen-printed carbon working electrode surface. Firstly, 3 µL of 1 µg mL^{-1} of anti-cytomegalovirus glycoprotein B capture antibody solution was deposited on the carbon working electrode surface and reacted two hours at 4ºC in a 100% moisture saturated environment. Subsequently, the excess of antibodies was washed with washing buffer (1% Tween 20 in 50mM pH 7.2 Tris-HNO_3 solution) and pH 7.2 Tris-HNO_3 solution. Next a drop of 3µL of blocking solution (BSA 5mg mL^{-1} in 50mM pH 7.2 Tris-HNO_3 solution) was applied and incubated for 60 minutes at room temperature, which was followed by washing again with washing buffer and pH 7.2 Tris-HNO_3 solution. The blocking solution blocks the possible remaining active sites and avoids non-specific adsorption [11,14].

To carry out the immunoreaction and electrochemical measurement, the immunosensor was firstly incubated, with 3 µL of a cytomegalovirus glycoprotein B solution (different concentrations), for 20 minutes, followed by washing it with washing buffer and pH 7.2 Tris-HNO_3 solution. Then, it was incubated with 3µL of AuNP-labeled anti-cytomegalovirus glycoprotein B solution for 20 minutes (under dark). After washing again with washing buffer and Tris-HNO_3 solution, 2µL of silver deposition solution was delivered on the surface of working electrode and incubated during 4 minutes (under dark), followed by rinsing with water. Finally, differential pulse voltammetry was performed from -0.06 V to 0.06 V in 1.0 M KCl solution to record the current.

II.4. Results and discussion

Has mentioned, the present proposed approach is based on a sandwich-type immunoassay, with the HCMV gB 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 attached AuNPs catalyzes the reduction reaction of silver ions, leading to the formation of AgNPs. Subsequently, the AgNPs suffer an oxidation process, by means of anodic stripping, and the analyte is quantified.

To achieve better analytical performance several parameters were optimized, namely the effect of silver deposition time on stripping analysis, the silver enhancer concentration, the antibody and BSA concentration, and the gB incubation time. Under optimal operation conditions, immunosensor performance was tested. Finally, in order to evaluate the feasibility, of the proposed immunosensor, for real sample analysis, the device was used for the determination of HCMV gB in spiked urine human samples, by using the standard addition method.

II.4.1. Optimization of Detection Conditions

II.4.1.1. Effect of Silver Deposition time on stripping current

Results for the effect of silver deposition time on stripping-current are displayed in figure II.2.. It can be seen that the stripping-current response increased greatly with the increasing silver deposition time until 4 minutes, after which it remained constant. Furthermore, after 6 min. of silver deposition, the electrochemical signal showed alterations (data not shown), thus 4 min was adopted as the optimal silver deposition time in this work.

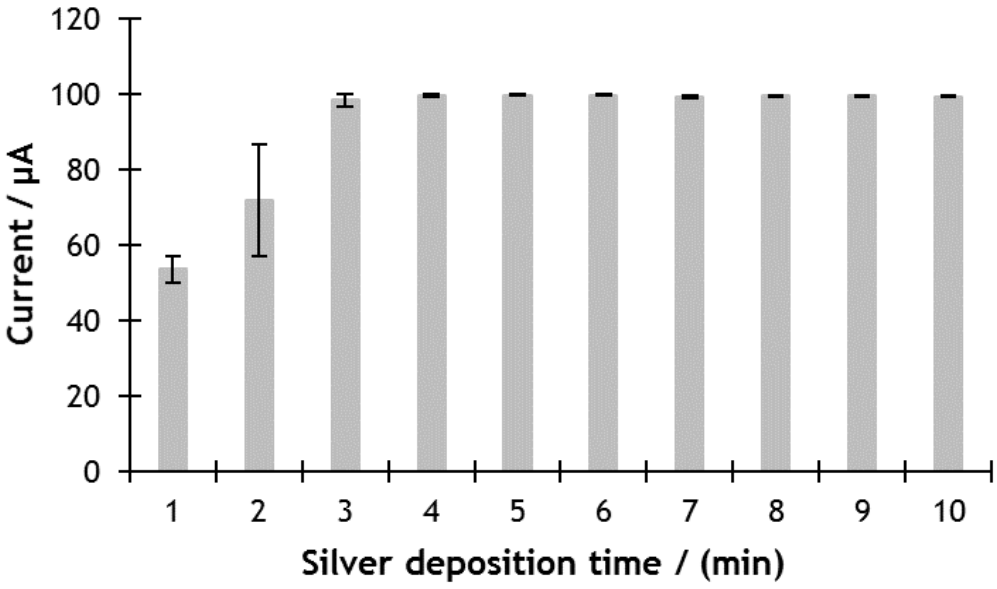

Figure II.2. Effect of silver deposition time on stripping-current response of AgNPs for 15 ng mL^{-1} gB in 1.0 M KCl. Results are the mean±SD of n=3 measurements.

II.4.1.2. Effect of Silver Enhancer Concentration

When running blank experiments (bare electrode) it was always observed the presence of a silver oxidation signal. In order to find the concentration where the AuNPs have the ability to amplify the signal with respect to the blank, different dilutions of silver enhancer solutions were tried. The oxidation signals of AgNPS onto the bare electrode surface and on the electrode modified with immobilized antibodies labeled with AuNPs were compared. It was observed that a 20-fold dilution of silver enhancer is a very concentrate solution, no difference was found in the signals intensity (figure II.3.a).

The best results, were reached in presence of a 140-fold dilution of the silver enhancer solutions (A and B) (figure II.3.b), accordingly this dilution was adopted as the optimal concentration for the silver enhancer.

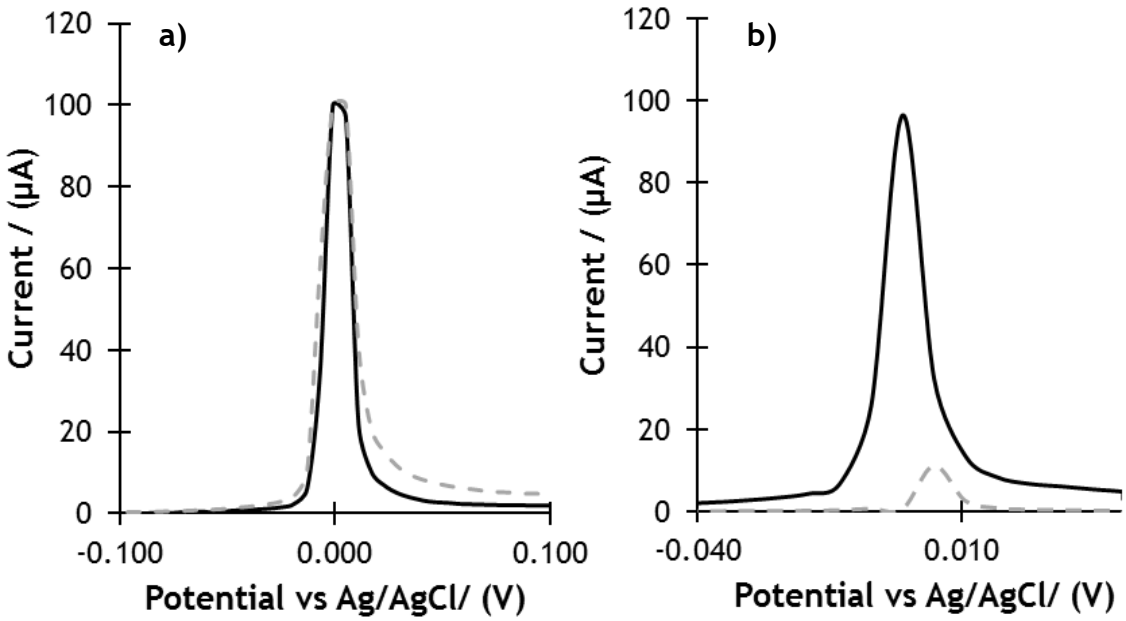

Figure II.3 Electrochemical signal intensity, for 15 ng mL^{-1} gB in 1.0 M KCl, at different concentrations of silver enhancer (study made in triplicate). (– – –) bare electrode; (———) electrode modified with Ab-AuNPS. a) 20-fold dilution; b) 140-fold dilution.

II.4.1.3. Effect of Antibody Concentration

The antibodies are one of the most expensive components used in the immunosensor construction, so in order to decrease the device cost without compromise its performance the effect of antibody amount used during immobilization, was studied. Five different antibody concentrations were tested (0.02; 0.125; 0.25; 0.5 and 1 µg mL^{-1}) using a concentration of 20 ng mL^{-1} gB and a 140 fold diluted silver enhancer solution, the obtained results are shown in figure II.4.

With the exception for 0.02 µg mL^{-1}, which presents the lowest current intensity, at all the other concentrations, current intensity is kept constant at approximately 27 µA. Nevertheless, we have chosen the 0.25 µg mL^{-1} concentration as the optimal one once it presents less dispersion.

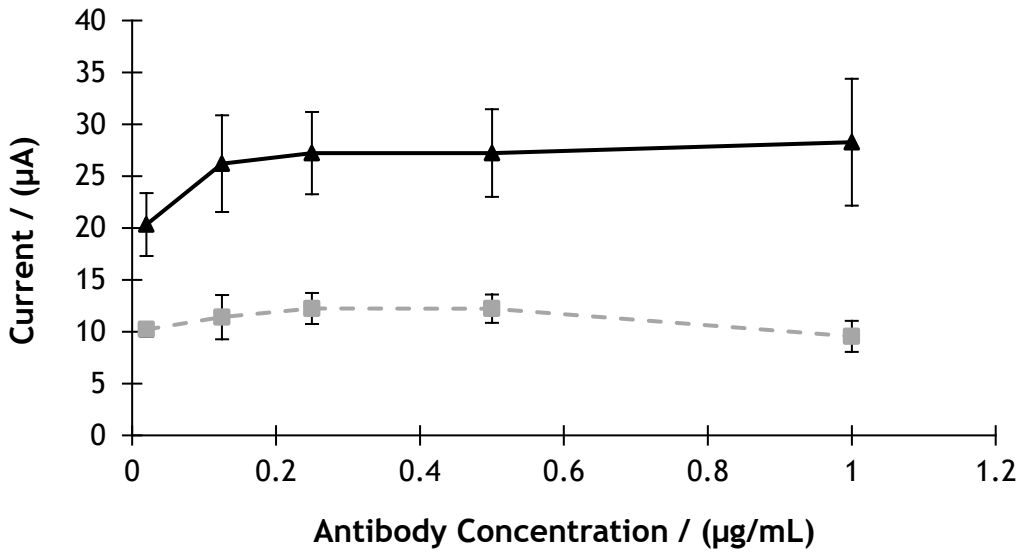

Figure II.4. Effect of antibody concentration on stripping-current response of AgNPs for 20 ng mL^{-1} of gB in 1.0 M KCl. Results are the mean±SD of *n=8* measurements. Blank; Assay

II.4.1.4. Effect of BSA Concentration

In order to avoid non-specific binding and improve the analysis, the effect of BSA (blocking agent) concentration on the stripping-current response was analyzed. The current intensity and signal dispersion of AgNPs for 20 ng mL^{-1} gB in 1.0 M KCl was studied in a range of BSA concentrations from 0 to 8 %.

The results (figure II.5.) exhibited a constant response from 2 to 6 %, after which current decreases probably due to the blockage of antigen binding sites on the immobilized antibody. Although, 2% seems to be the optimal BSA concentration, all of our experiments were performed using a concentration of 5%. Nevertheless, this cannot be considered an issue, since this concentration is integrated in the constant current response zone.

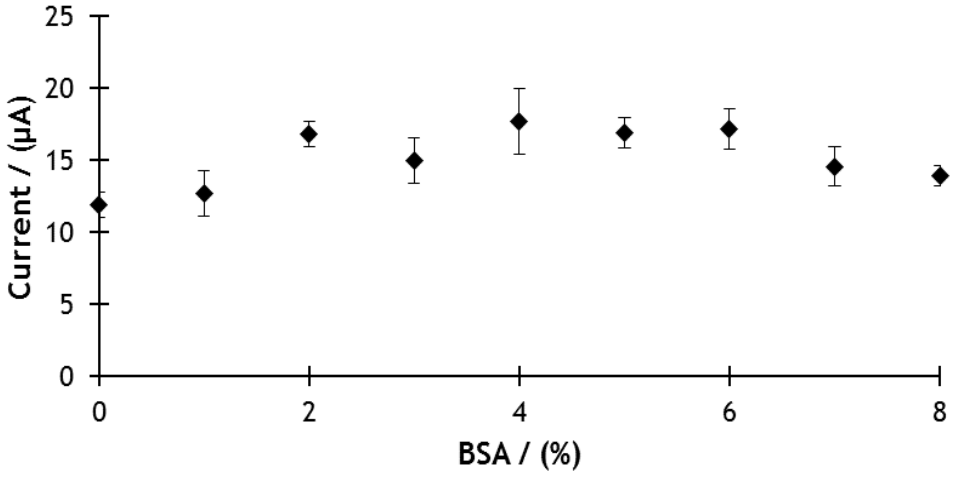

Figure II.5. Effect of BSA concentration on stripping-current response of AgNPs for 20 ng mL^{-1} gB in 1.0 M KCl. Results are the mean±SD of *n=8* measurements.

II.4.1.5. Effect of gB Incubation Time

The time used for the complexation reaction between antigen and antibody is an important step affecting the analytical performance of the immunosensor. This optimization can reduce the time needed to complete the assay. Incubations of 20 ng mL^{-1} of HCMV gB, during 0, 10, 20, 30, 40, 50 and 60 minutes were carried out.

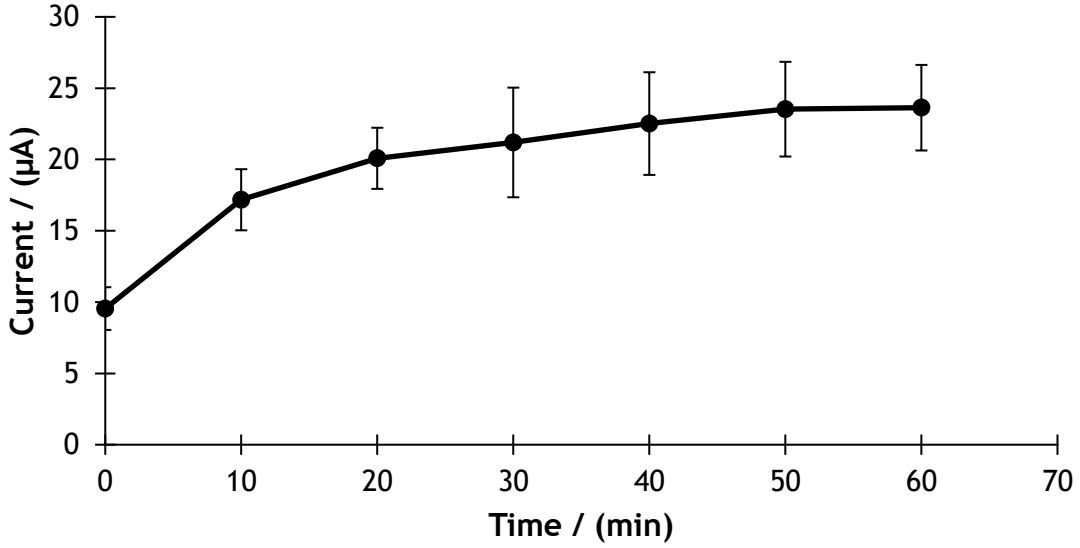

Figure II. 6. Effect of incubation time on stripping-current response of AgNPs for 20 ng mL^{-1} gB in 1.0 M KCl. Results are the mean±SD of *n=8* measurements.

The evaluation of the stripping voltammetric response (figure II.6.) indicated that after 20 minutes, current intensity is maintained constant, showing saturation in the formation of

the sandwich immunocomplex [11]. Once, 20 minutes presents the highest current intensity with the lowest standard error (figure II.6.), this incubation time was selected to run the immunoassay.

II.4.2. Immunosensor performance

With the presented sandwich-type immunoassay format, the quantitatively deposited AgNPs could be easily detected by anodic stripping voltammetric analysis in KCl solution. In figure II.7.a) we can observe the change in stripping current as function of gB concentration.

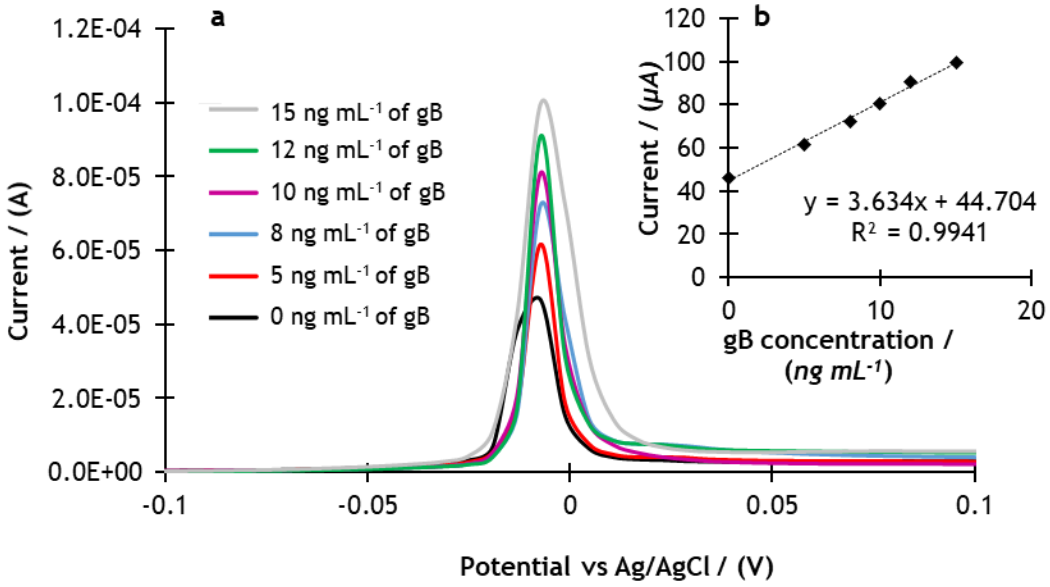

Figure II.7. Linear-sweep stripping voltammetric curves of AgNPs a) and calibration curve b) for gB detection using the proposed strategy. gB was diluted in Tris-HNO_3 buffer. a) Curves are the differential pulse voltammetry responses for gB at concentrations from 0 to 15 ng mL^{-1}.

Current increases linearly with analyte concentration (figure II.7.b) in the range from 5 ng mL^{-1} to 15 ng mL^{-1}. Calibration curves (made in triplicate) (table II.1.) showed good linear correlation coefficients between current intensity and gB concentration.

To test the biological matrix effect on the immunosensor, a 2-fold diluted urine sample (prepared in 50 mM Tris HNO_3 buffer pH 7.2) from a healthy patient was used to obtain solutions with different gB concentrations. As observed with gB samples prepared in buffer, the ones prepared in the biological matrix also exhibited a linear dependence between analyte concentration and corresponding anodic stripping current intensity (figure II.8.).

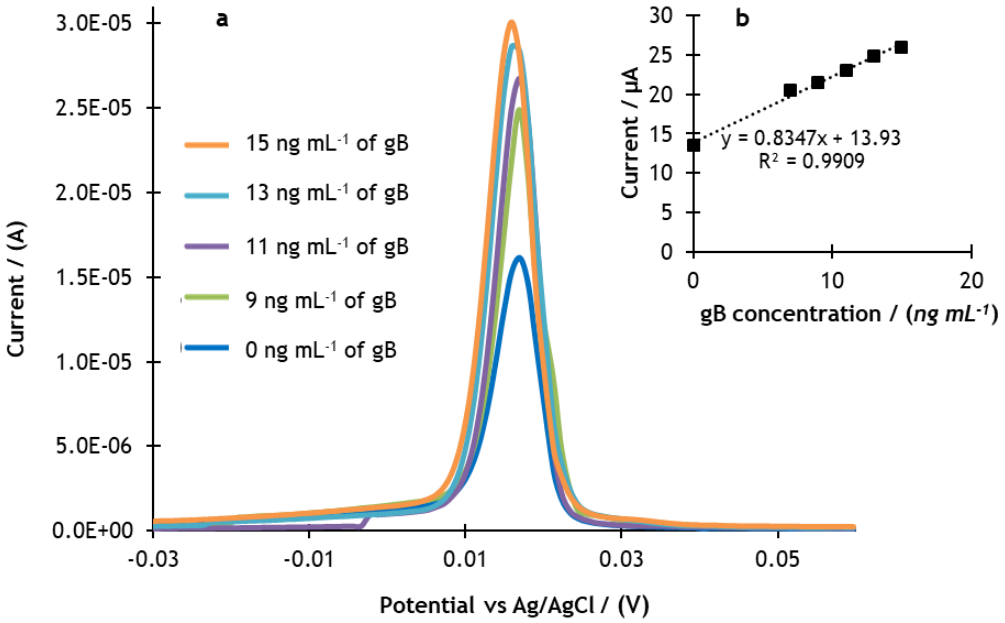

Figure II.8. Linear-sweep stripping voltammetric curves of AgNPs a) and calibration curve b) for gB detection using the proposed strategy. gB was diluted in urine samples diluted 1:2 in tris-HNO_3 buffer. a) Curves are the differential pulse voltammetry responses for gB at concentrations from 0 to 15 ng mL^{-1}.

Calibration curves (made in triplicate) (table II.1.) likewise showed good linear correlation coefficients.

Table II.1. Calibrations parameters for gB prepared in buffer and urine samples (1:2 dilution). RSD of analyte detection on different electrodes. i.e. it shows reproducibility of construction of independent biosensor devices.

Matrix	Equation	Slope	R^2	RSD (%)	Detection Limit (ng mL^{-1})
Tris-HNO_3 buffer	y=4.7x+28.0	4.7	0.98	13.5	3.3 ± 1.7
	y=4.6x+65.1	4.6	0.99		
	y=3.6x+44.7	3.6	0.99		
Urine	y=0.7x+8.0	0.7	0.99	11.2	3.2 ± 0.2
	y= 0.9x+17.8	0.9	0.99		
	y=0.8x+13.9	0.8	0.99		

The relative standard deviation (RSD) associated with the calibration curves slopes for samples prepared in Tris-HNO_3 buffer and diluted urine were respectively 13.5% and 11.2% (table II.1.). Although high, these values are consistent with the ones obtained for some immunosensors [15]. The immunosensor mode of preparation (direct adsorption of the capture antibody in the working electrode) may also have affected immunoassay observed results. By physical adsorption, antibodies are randomly adsorbed onto the working electrode surface, being some antigen-binding regions blocked and the biosensor performance reduced [16].

In the analytical field, the ability to obtain the lowest limit of detection (LOD) is the most critical feature. The first stage in the LOD calculation was the determination of a linear relation between gB concentration and current signal as shown in figure II.7.b), figure II.8.b)

and table II.1. The incorrect adjustments due to the existence of anomalous points, were avoided using least median squares regression [17,18]. Then, in order to check the technique LOD value, results from three calibration curves were evaluated using the DETARCHI program [19], a program that calculates detection limits with an evaluation of the probability of false positive (α) and negative (ß), according to ISO11843-2, 2000 [20]. An average limit of detection of 3.3 ± 1.7 ng mL^{-1} for HCMV gB in buffer and 3.2 ± 0.2 ng mL^{-1} for HCMV gB in urine samples was obtained for α=ß=0.05 (Table 5.1.). These two values are similar, leading us to consider that the matrix does not interfere with the immunosensor response.

Finally, our results were compared with the ones obtained by Susmel *et al.* [21]. In their studies a piezoelectric immunosensor for cytomegalovirus gB detection was developed. The immunosensor provided a detection limit of 1µg mL^{-1} and offered a linear range from 2.5 to 5 µg mL^{-1}. Comparatively, the present electrochemical immunosensor offers a lower LOD being able to detect trace concentrations.

II.4.3. Application in the analysis of spiked urine human samples

In order to evaluate the analytical reliability of the proposed immunosensor for real sample analysis, the immunosensor was used for the determination of gB in urine samples. Three urine samples collected from healthy humans were spiked with respectively 4, 6 and 7 ng mL^{-1} of gB. By application of the standard addition method, the initial concentrations of gB added to each urine sample was obtained (table II.2).

Table II.2. Results of HCMV gB determination in urine samples by application of the standard addition method. (N is the number of experimental data points).

HCMV gB concentration (ng mL^{-1})	N	Standard addition method equation	R^2	Detected content (ng mL^{-1})	Relative error (%)
4.00	5	y=0.67x+2.91	0.99	4.32 ± 0.62	8.1
6.00	4	y=0.73x+3.87	0.99	5.30 ± 0.71	-11.7
7.00	4	y=0.78x+6.25	0.99	8.00 ± 0.78	14.3

A value of 4.32 ± 0.62 ng mL-1 was found for the lowest gB concentration (relative error 8.1%), a value of 5.30 ± 0.71 ng mL^{-1} was found for the sample spiked with 6 ng mL^{-1} of gB (relative error -11.7%) and a value of 8.00 ± 0.78 ng mL^{-1} was found for the highest added gB concentration (relative error 14.3%).

The observed relative error values seem to be in accordance with the ones usually presented for these type of immunosensors [11,15]. For HCMV detection, no standard method is available to compare with this electrochemical immunosensor, since it uses the gB detection

and other currently used techniques are based on DNA amplification, antibody screening or virus isolation in fibroblast culture (the gold standard method for HCMV detection) [3]. Nevertheless, the overall analysis time of the proposed biosensor is considerably shorter when compared to usually applied methods: shell-vial (approximately 24 hours), polymerase chain reaction (approximately 6 hours) and ELISA assay (at least 4 hours).The constructed biosensor can analyze a sample within 50–60 min with 20 min needed for gB incubation plus more 20 min for AuNP-labeled anti-cytomegalovirus gB incubation, about 5 minutes for the anodic stripping voltammetry and a couple of minutes necessary for the electrode washing and the electrode integration within the measurement set-up. In terms of selectivity, as in the case of ELISA tests, the presented immunosensor also presents the possibility of false positive results, caused by cross-reactions with some virus of *Herpesviridae* family, rheumatoid factors and antinuclear antibodies [22].

II.5. Conclusions

Established in screen-printed electrodes and with help of nanoparticles, it is proposed an inexpensive, simple and disposable electrochemical sandwich type immunosensor for gB detection in urine. The basic principle is that a higher concentration of HCMV gB means a higher amount of captured gold nanoparticles on the sensor surface, resulting in higher silver deposition with higher measured signals. This study achieved variations in the stripping-current response that directly correlated with the variation in HCMV gB concentration. Detection conditions have been optimized. A saturation profile was reached for 15 ng mL^{-1} of HCMV gB. The calculated detection limit in urine was 3.2 ± 0.2 ng mL^{-1} of HCMV gB, which greatly improves the one obtained by Susmel *et al.* [21] in the detection of the same protein using a piezoelectric biosensor. The reproducibility, evaluated by the RSD, obtained from calibration curves of different assays, was considered acceptable for this type of biosensor (11.2 %) [15]. Application of the biosensor in the analysis of spiked urine human samples, provided results with relative errors less than 14.3%, indicating that the proposed method accuracy must be improved for detection HCMV gB in clinical samples. Nevertheless, it was demonstrated the possibility of using an electrochemical based immunosensor for the rapid detection of gB in human urine samples (approximately 1 hour). These promising results provide excellent guidelines for the device improvement. Affecting the reproducibility of the immunoassay is a deficiency on the antibodies immobilization. As mentioned, by physical adsorption, antibodies are randomly adsorbed onto the working electrode. Ongoing studies are trying different antibody immobilization strategies in order to improve the immunosensor performance.

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Bibliography

1. Revello, M. G. & Gerna, G. Diagnosis and Management of Human Cytomegalovirus Infection in the Mother, Fetus, and Newborn Infant. *Clin. Microbiol. Rev.* **15**, 680–715 (2002).

2. Domingues, F., Sousa, F. & Queiroz, J. A Biotecnologia no Diagnóstico do Citomegalovírus Humano. *Bol. Biotecnol.* **82**, 28–35 (2005).

3. Yen-Lieberman, B. Diagnosis of Human Cytomegalovirus Disease. Clin. Microbiol. Newsl. **22**, 105–109 (2000).

4. Ferreira, S., Queiroz, J. & Domingues, F. Production of a Fusion Protein Containing the Antigenic Domain 1 of Human Cytomegalovirus Glycoprotein B. J. Microbiol. Biotechnol. 16, 1026–1031 (2006).

5. Renedo, O. D., Alonso-Lomillo, M. A. & Martínez, M. J. A. Recent developments in the field of screen-printed electrodes and their related applications. *Talanta* **73**, 202–219 (2007).

6. Zhang, S., Wright, G. & Yang, Y. Materials and techniques for electrochemical biosensor design and construction. *Biosens. Bioelectron.* **15**, 273–282 (2000).

7. Ghindilis, A. L., Atanasov, P., Wilkins, M. & Wilkins, E. Immunosensors: electrochemical sensing and other engineering approaches. *Biosens. Bioelectron.* **13**, 113–131 (1998).

8. Warsinke, A., Benkert, A. & Scheller, F. W. Electrochemical immunoassays. J. *Anal. Chem.* **366**, 622–634 (2000).

9. Speckner, A., Glykofrydes, D., Ohlin, M. & Mach, M. Antigenic domain 1 of human cytomegalovirus glycoprotein B induces a multitude of different antibodies which, when combined, results in incomplete virus neutralization. *J. Gen. Virol.* **80**, 2183–91 (1999).

10. Muñiz, A., Parolo, C. & Merkoçi, A. Immunosensing using nanoparticles. *Mater. today* **13**, 24–34 (2010).

11. Lai, G., Wang, L., Wu, J., Ju, H. & Yan, F. Electrochemical stripping analysis of nanogold label-induced silver deposition for ultrasensitive multiplexed detection of tumor markers. *Anal. Chim. Acta* **721**, 1–6 (2012).

12. Lai, G., Yan, F. & Ju, H. Dual signal amplification of glucose oxidase-functionalized nanocomposites as a trace label for ultrasensitive simultaneous multiplexed electrochemical detection of tumor markers. *Anal. Chem.* **81**, 9730–9736 (2009).

13. Bi, S., Yan, Y., Yang, X. & Zhang, S. Gold nanolabels for new enhanced chemiluminescence immunoassay of alpha-fetoprotein based on magnetic beads. *Chem. an Eur. J.* **15**, 4704–4709 (2009).

14. White, N. M. & Turner, J. D. Thick-film sensors: past, present and future. *Meas. Sci. Technol.* **8**, 1–20 (1997).

15. Bertok, T. *et al.* Label-free detection of glycoproteins by the lectin biosensor down to attomolar level using gold nanoparticles. *Talanta* **108**, 11–18 (2013).

16. Trilling, A. K., Beekwilder, J. & Zuilhof, H. Antibody orientation on biosensor surfaces: a minireview. *Analyst* **138**, 1619–1627 (2013).

17. Massart, D. L. & Kaufman, L. Least median of squares: a robust method for outlier and model error detection in regression and calibration. *Anal. Chim. Acta* **187**, 171–179 (1986).

18. Rousseeuw, P. J. & Leroy, A. M. Robust Regression and Outlier Detection. J. *Appl. Econom.* **4**, 309–311 (1989).

19. Sarabia, L. & Ortiz, M. C. DETARCHI: A program for detection limits with specified assurance probabilities and characteristic curves of detection. *Trends Anal. Chem.* **13**, 1–6 (1994).

20. ISO 11843-2: Capability of detection - Methodology in the linear calibration case. (2000).

21. Susmel, S., O'Sullivan, C. & Guilbault, G. Human cytomegalovirus detection by a quartz crystal microbalance immunosensor. *Enzyme Microb. Technol.* **27**, 639–645 (2000).

22. Daiminger, A., Bäder, U., Eggers, U., Lazzarotto, T. & Enders, G. Evaluation of two novel enzyme immunoassays using recombinant antigens to detect cytomegalovirus-specific immunoglobulin M in sera from pregnant women. *J. Clin. Virol* **13,** 161–171 (1999).

CHAPTER III

A rapid magnetic particle-based enzyme immunoassay for human cytomegalovirus glycoprotein B quantification

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A rapid magnetic particle-based enzyme immunoassay for human cytomegalovirus glycoprotein B quantification

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Keywords

Magnetic beads; immunoassay; mpEIA; Human cytomegalovirus; Glycoprotein B

III.1. Summary

Human cytomegalovirus (HCMV) is a herpes virus that can cause severe infections. Still, the available methods for its diagnostic have the main disadvantage of requiring long time to be performed. In this work, a simple magnetic particle-based enzyme immunoassay (mpEIA) for the quantification of glycoprotein B of Human cytomegalovirus (gB) in urine samples is proposed. The immunosensor scheme is based on the analyte protein gB sandwiched between a primary monoclonal antibody, (MBs-PrG-mAb1), and a secondary anti-gB antibody labelled with *Horseradish peroxidase* (Ab2-HRP) to allow spectrophotometric detection.

The mpEIA analytical performance was tested in urine samples, showing a linear dependence between gB concentration and the absorbance signal at 450 nm in a range of concentrations from 90 to 700 pg mL^{-1}. The calculated detection limits for gB were 90±2 pg mL^{-1} and the RSD was about 6.7% in urine samples. The immunosensor showed good selectivity against other viruses from Herpesviridae family, namely varicella zoster and Epstein Barr viruses. The recoveries of spiked human urine samples at 0.30-0.50 ng mL^{-1} concentration levels of gB ranged between 96 to 104%. The proposed mpEIA method was validated following the guidelines of the European Medicines Agency (EMEA-2014), and allows rapid, successful and easy quantification of gB in urine samples.

III.2. Introduction

Human cytomegalovirus (HCMV) is one of the eight herpesviruses that commonly infect humans, being its transmission facilitated by mucous contact [1,2]. It is recognized as the most common congenital viral infection in human and an important cause of morbidity and mortality in immunocompromised hosts [3]. Primary infection by HCMV results in the establishment of a lifelong latent infection, and its reactivation may occur in situations of immune system dysfunction or low maturity, resulting in severe clinical symptoms [1,2,4,5]. Still, the diagnosis of HCMV remains controversial because of the difficulty in separating patients who are asymptomatic (but carry HCMV) from patients who have the symptomatic disease [1]. The standard for diagnosis of HCMV infection are serological tests based on IgM and IgG detection [6,7]. IgG detection is only valuable for establishing past infection, or confirming seroconversion, while IgM has poor sensitivity and specificity to detect a recent infection. These tests usually require a minimum of two days [3]. On the other hand, the traditional method for direct free HCMV detection is viral isolation that detect viral antigens in tissue, urine or saliva samples. Nevertheless, time analysis requirements for this method (≈ 20 days) makes it disadvantageous to be routinely used in clinical diagnosis as point of care [8,9].

As an alternative, the use of capture antibodies against the envelope glycoproteins of HCMV open the possibility of faster immunochemical methods of analysis. Glycoprotein B of

HCMV (gB) is a viral glycoprotein that plays a crucial role in virus entry into the cell and emerges during the early stages of a HCMV infection [10]. In addition, gB is the dominant antigen in the envelope of HCMV, being possible its determination in body fluids like urine, where viral loads are higher [11,12]. This is quite advantageous since the collection of the biological samples is simple and is performed by noninvasive procedures. In consequence, the development of new methods for the accurate detection of gB in body fluids, is of great interest, unlike the up mentioned analytical method based on the determination of antibodies generated by the HCMV virus [8,12].

Enzyme-linked immunosorbent assays (ELISA) have also great presence in clinical diagnostic tests [13,14]. However, conventional ELISAs have some limitations, such as high costs (e.g. high quantities of antibodies immobilized on the wells of the microtiter wells), slow kinetics, and nonspecific adsorptions of conjugates and/or matrix interferers on the microplate wells [14]. Much of these limitations have been greatly improved with the recent use of surface-functionalized magnetic micro-beads (MBs), in the so-called magnetic particle-based enzyme immunoassays (mpEIA) [14]. The use of MBs improves the affinity interaction thanks to a faster assay kinetics of the dispersed beads. The surface area is higher than a flat solid phase of the wells (in a classical microtiter plate), and the washing and separation efficiencies are enhanced by using an external well-controlled magnetic field, allowing the analysis of complex samples without any pre-enrichment or purification steps [14-16].

Thus, in this work, we propose a spectrophotometric mpEIA method for the rapid and simple gB determination in urine samples (see the immunoassay scheme of figure III.1.). Magnetic particles functionalized with protein G (MBs-prG), allows the antibody (mAb1) oriented immobilization, resulting in a more effective recognition of gB [17]. The spectrophotometric detection was performed through a secondary antibody labelled with *Horseradish peroxidase* (Ab2-HRP) [8,10,14,18]. Special attention was given to the non-specific adsorptions (NSA) of gB and Ab2-HRP, as they have a very negative influence in quantitative immunoassays [19]. After optimization of the experimental conditions, the validation of the mpEIA method was carried out according to the "Guideline on bioanalytical method validation", from the European Medicines Agency (EMEA) (2014) [20]. The developed immunosensor was shown to be a fast and effective method for gB quantification in human urine samples for the useful determination of HCMV infections.

III.3. Material and methods

III.3.1. Apparatus

Spectrophotometric measurements were made with a Bio-Rad model 680 Microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, CA, USA). Standard 96-well polystyrene microplates (ref. 82.1581) were supplied from Sarstedt (Nümbrecht, Germany). A magnetic 96-well separator (ThermoFisher Scientific, Waltham, MA, USA) was used to separate the MBs from the supernatants to make easier the removal of solvent from the standard ELISA plates. Incubation process was carried out using Grant Bio POS-300 Orbital Shaking Platform (Cambridge, UK). A magnetic separation stand for eppendorf vials (Z5342, 12 positions, 1.5 mL volume) was purchased from Promega (Madison, WI, USA).

III.3.2. Reagents

Human cytomegalovirus glycoprotein B (gB) and Anti-Cytomegalovirus glycoprotein B primary and secondary antibodies (mAb1 and Ab2-HRP) were purchased from Abcam (ab43040, ab69245, ab6499, respectively) (Cambridge, UK). Bovine serum albumin (BSA), peroxidase substrate 3,3',5,5'-tetramethylbenzidine liquid substrate system for ELISA (TMB), Tween-20 and monosodium phosphate were obtained from Sigma-Aldrich (Madrid, Spain). Disodium phosphate and sulfuric acid were acquired from Panreac (Barcelona, Spain). Sodium chloride was purchased from Fisher Scientific (Bishop's Stortford, UK) and dynabeads® protein G immunoprecipitation kit and Pierce Protein-Free were products from Thermofisher Scientific. Urine samples were obtained from healthy volunteers after informed consent. All the reagents used were of analytical grade and Milli Q water Millipore (Burlington, MA, USA) was used for preparing all solutions.

III.3.3. Primary antibody immobilization on functionalized magnetic beads

Dynabeads® were re-suspended in the vial (vortexed >30 sec or tilted and rotated for 5 min). Then, 50 µL (1.5 mg) of MBs-PrG Dynabeads® were transfer to a tube, washed three times with 500 µL of PBS buffer in order to remove NaN_3 preservative and the surfactant. Each

washing step consisted on the re-suspension of the beads in PBS buffer, followed by supernatant clearance under a magnetic field that retain the beads and separate them from solution.

After MBs-PrG cleaning, the desired amount of primary antibody (mAb1), diluted in PBS, was added to them and left to incubate for 10 min at room temperature. The antibody modified MBs (MB-PrG-mAb1) were washed three times with PBS buffer, reconstituted in PBS (1.0 mL), and stored at 4 ºC when not in use. According to the manufacturer, it is not recommended to freeze (−20 °C) these kind of MBs.

III.3.4. Immunoassay determination of gB

All the immunoassay incubations were made on standard polystyrene flat-bottom microtest plates coupled with a magnetic support with 96x individual magnets to hold back the modified MBs on each of the microplate wells. In order to avoid nonspecific adsorption, the wells of the microplate were, firstly, blocked overnight by using the commercial solution Pierce Protein-Free. Following emptying of the wells, 25 µL of MBs-mAb1 (containing 5 µg of MBs) were added to each well and the remaining sites of the beads surface blocked with BSA 4% during 60 minutes at room temperature. After this, 25 µL of gB standard (or the clinical sample) were added to obtain a final concentration over the range from 0 to 800 pg mL^{-1} gB. Incubations were carried out for 60 minutes at room temperature with gentle orbital agitation. Finally, 25 µL of the Ab2-HRP was added to each well and left to incubate for 60 minutes at room temperature with gentle orbital agitation.

Between each of the incubation steps, the wells were washed three times with 300 µL of PBS and the solvent removed under an external magnetic field for retaining the modified MBs. For the mpEIA spectrophotometric measurements of Ab2-HRP, 50 µL of the peroxidase substrate TMB reagent was added to each well, left to react for 10 min at room temperature, stopped with 0.5 M H_2SO_4 and the absorbance measured at 450 nm with an ELISA reader.

III.4. Results and Discussion

As it is well known, immunoassays are greatly dependent on experimental conditions, being imperative their detailed optimization for achieving the best analytical properties. A sandwich assay scheme was used for the construction of our immunosensor, as it improves selectivity against a competitive immunoassay scheme, once two selected antibodies are used for the specific recognition of different parts of the same gB protein. After incubation with the primary antibody, a HRP-labeled secondary antibody (Ab2-HRP) was used to generate the

analytical signal for gB detection. The scheme of this mpEIA immunosensor assay is shown in figure III.1.

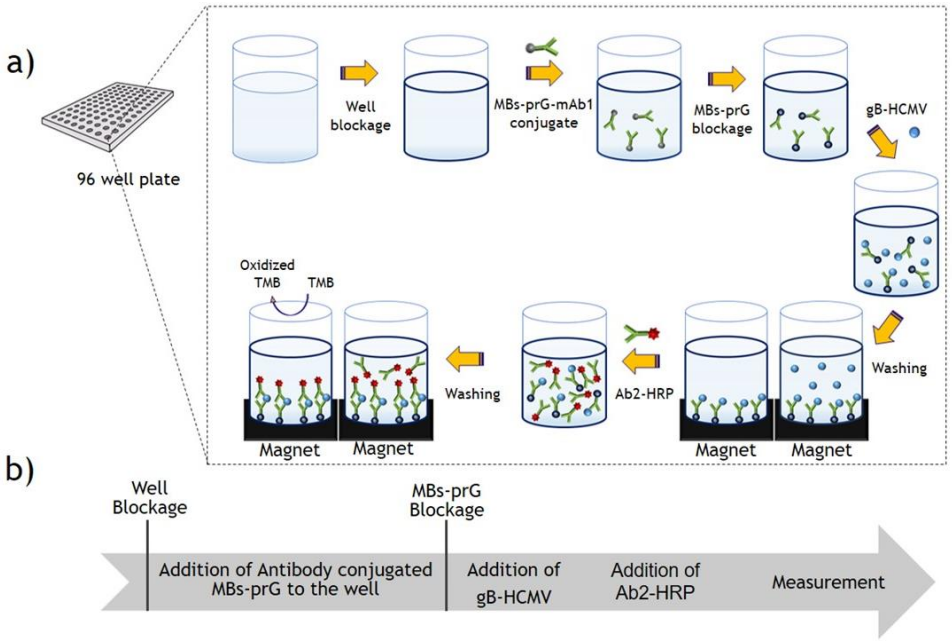

Figure III. 1. a) Schematic representation of the mpEIA sandwich assay method. Mbs-PrG-mAb1; gB; Ab2-HRP b) Identification of the steps where blockage is needed.

Special attention was paid to avoid NSA of the gB and the Ab2-HRP conjugated antibody, both on the wall of the microtiter wells and on the modified surface of MBs-PrG-mAb1, since this would falsify the analytical signals, not due to the extension of the incubation reactions. It was also considered the possibility of unwanted interactions between primary and secondary antibodies [21].

After optimizations, the mpEIA analytical performance in quantifying gB was tested. In order to evaluate the feasibility of the proposed assay for real samples analysis, the mpEIA was used for the determination of gB in human urine samples, and cross-reactivity evaluated with Epstein-Barr virus (EBV) and Varicela-zoster (VZV) antigens, both viruses from the *Herpesviridae* family.

III.4.1.Optimization of the experimental conditions

It is well known that NSA can occur on the surface of the 96 well polystyrene ELISA plate through physical adsorption by electrostatic and/or van der Waals forces and/or by hydrophobic interactions [22]. In order to avoid NSA of the biological reagents on the wells, three blocking agents were tested, namely the SuperBlock and Pierce Protein-Free commercial solutions and a 4% bovine serum albumin (BSA) solution prepared in 0.1 PBS (pH=7.4). After blocking the wells overnight with these solutions, incubations of the secondary Ab2-HRP

antibody were carried out (25 µL for 60 min.), and the absorbances measured after reaction with TMB, as explained in section III.3.4. The results are shown in figure III.2.

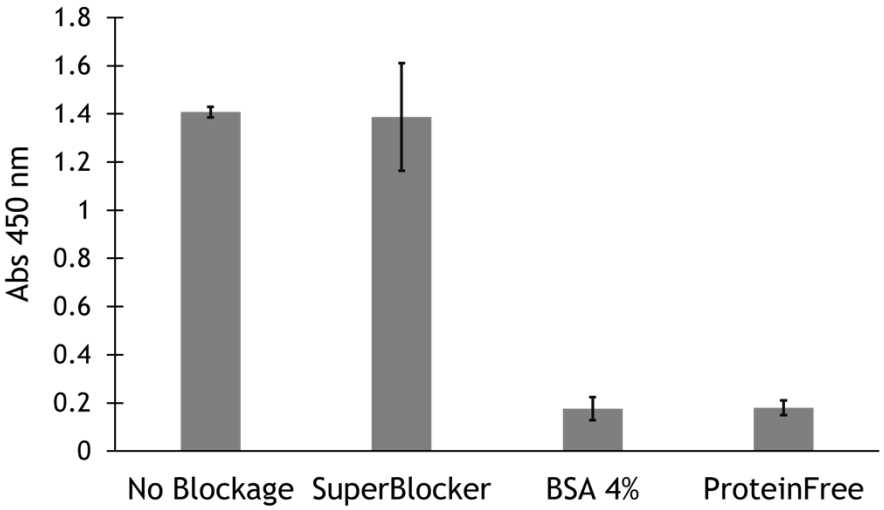

Figure III. 2. Absorbances measured after the well blockage with SuperBlock, Pierce ProteinFree and BSA blocking agent solutions, after incubations with Ab2-HRP for 60 min. The absorbance values are the mean±SD of n=5 replicates.

As it can be seen, in presence of SuperBlock no difference is observed compared to when no blockage is performed, demonstrating secondary antibody nonspecific adsorption to the well's walls and the inefficiency of this blocking agent. The lowest absorbance values were obtained with 4% BSA and Pierce Protein-Free solutions. Despite the similarity between their absorbance mean values (0.175 and 0.180 respectively), Pierce Protein-Free solution was chosen as the optimal blocking agent, once the relative standard deviations obtained in replicated tests were of 17.3% for Pierce Protein-Free and 27.4% for BSA blocking solutions.

Although hydrophobic interactions play an essential role in epitope-antibody binding, these forces may also promote nonspecific interactions between primary and secondary antibodies. Low levels of a detergent, such as Tween-20, are known to reduce surface tension and as consequence this type of interaction [23]. Accordingly, in order to further lower the background signal, a PBS + 0.02% Tween-20 solution was used during the secondary antibody incubation step. No changes of the absorbances were observed using tween-20 solutions, showing that hydrophobic interactions do not significantly contribute to the global NSA (figure III.3.).

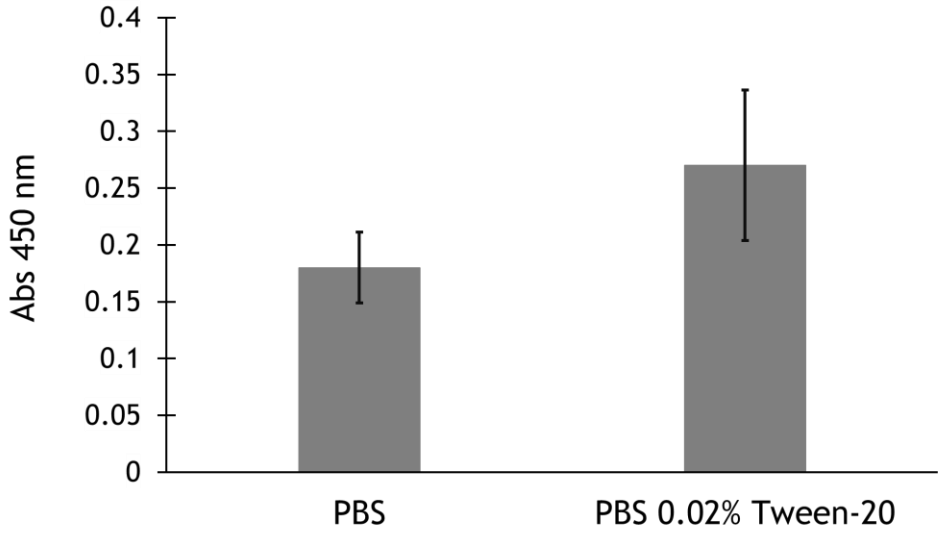

Figure III. 3. Comparison between the use of PBS and PBS with 0.02% tween 20 during Ab2-HRP incubation and study of tween 20 effect in nonspecific antibody binding. The absorbance values are the mean±SD of n=5 replicates.

With the use of MBs modified with capture antibody in the immunosensor scheme, a high background signal was observed after incubations with Ab2-HRP (without gB), indicating nonspecific adsorptions of the conjugated Ab2-HRP antibody on the unblocked MBs surface. Thus, the MBs blockage was considered to avoid this problem [24,25]. Two blocking agents (BSA and Pierce Protein-Free) were evaluated. The lower absorbance signals due to NSA of Ab2-HRP were obtained with 4% and 5% of BSA solutions, as it is shown in figure III.4..

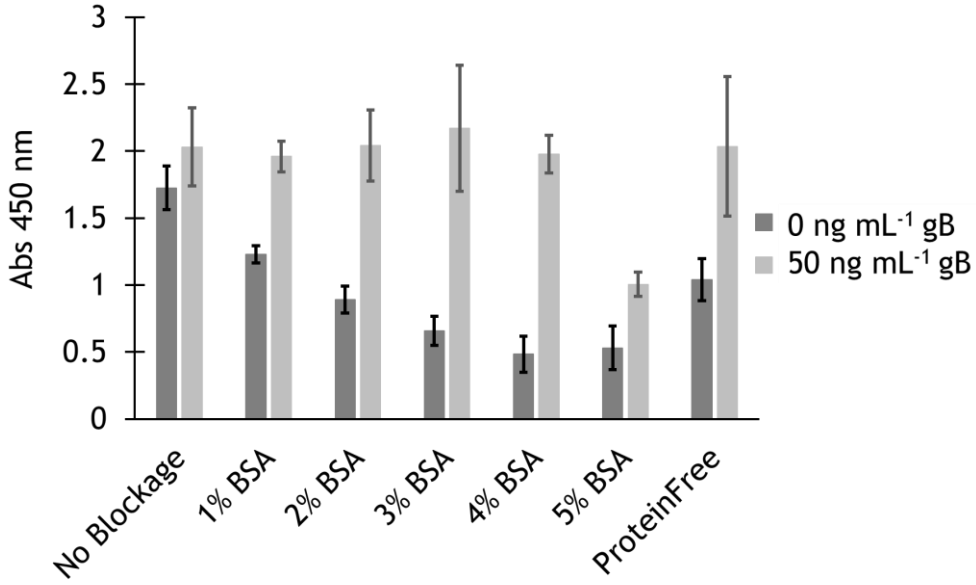

*Figure III. 4.*Influence of the MBs-PrG-mAb1 blockage on background absorbances from NSA of Ab2-HRP and on absorbances from gB detection. An excess of concentration of 50 ng mL^{-1} gB was used. Results are the mean±SD of n=5 replicates.

However, a decrease in gB detection absorbances were found with more than 5% of BSA solutions, suggesting that BSA blocks some antigen binding sites of the mAb1 antibody, reducing the ability to detect gB. The best concentration for this purpose was 4% BSA, once it shows lower blank signals and it did not interfere with the gB antigen detection. Experimental conditions strongly influence the sensitivity of the sandwich immunoassays. In particular, it is important that the number of available binding sites of the primary (mAb1) and secondary (Ab2-HRP) antibodies be in excess of the stoichiometric amount of the gB analyte. Under these conditions, adequate absorbance values are produced (Abs ≤ 1.0) according to figure III.5. a linear range of concentrations from 70 pg mL^{-1} to 800 pg mL^{-1} was obtained.

III.4.2. Immunosensor analytical performance

The analytical performance of the immunoassay was evaluated following the guidelines of the European Medicines Agency on bioanalytical method validation [20]. Accordingly, the main characteristics that are essential to ensure the acceptability of a bioanalytic method are: calibration curve performance, lower limit of quantification, matrix effect, precision, selectivity and accuracy.

Calibration standards were prepared measured in PBS buffer and urine matrices for comparison purposes. Urine was chosen to evaluate the immunosensor performance, once high virus load is found in excreted urine from HCMV infected individuals [11,12]. In presence of PBS buffer, absorbance at 450 nm increased linearly with analyte concentration in the narrow range from 70 pg mL^{-1} to 800 pg mL^{-1}, showing the calibration curve a good linear correlation coefficient (table III.1). Higher concentrations produced saturation of the binding sites of primary antibody. Although this range of concentrations quantification is small, it provides clinically useful and sensitive information for the control of HCMV, once the appropriate dilution of the samples has been carried out (figure III.5.).

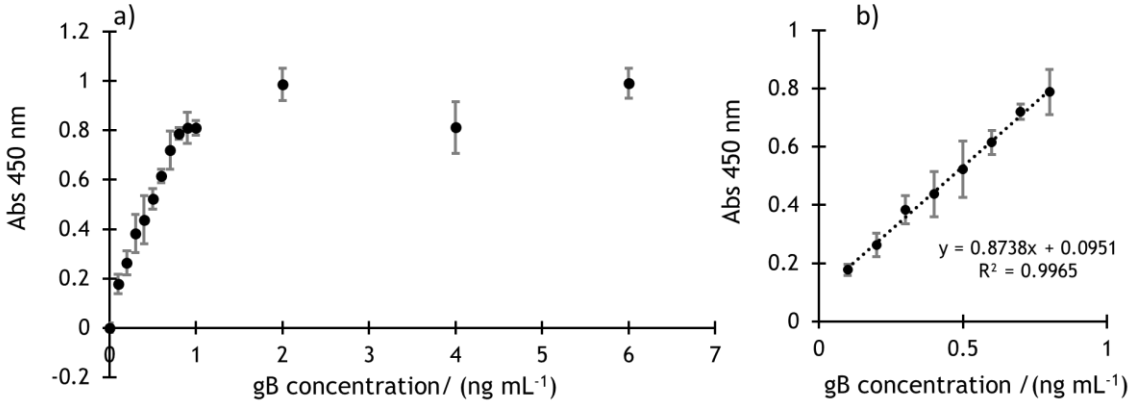

Figure III. 5. Absorbance signals of the mpEIA assay method for gB concentrations from 0.00 to 6.000 ng mL-1 in 0.1 M. buffer matrix. a) and calibration curve from 0.07 to 0.80 ng mL-1 b). Experimental conditions: 5 µg of saturated with antibody MBs added to the wells (8 µg of mAb1 per mg of MBs-PrG). Results are the mean±SD of n=3 replicates.

To test the biological matrix effect on the magnetic particles-based immunoassay, gB standards were prepared in a 2-fold diluted urine sample from healthy patients in PBS buffer pH 7.4. Replicated calibration graphs were carried out in PBS 0.1 M. buffer and in diluted urine samples. The linear range in presence of urine (between 90 pg mL^{-1} to 700 pg mL^{-1} of gB) (figure III.6.) was slightly smaller than that observed in PBS buffer, possibly due to the presence of other matrix components in the urine samples, that might block some antigen binding sites of the primary mAb1, leading to an earlier saturation. Nevertheless, there was not significant differences between the slopes of both calibrating graphs in the two matrices (95% level of confidence).

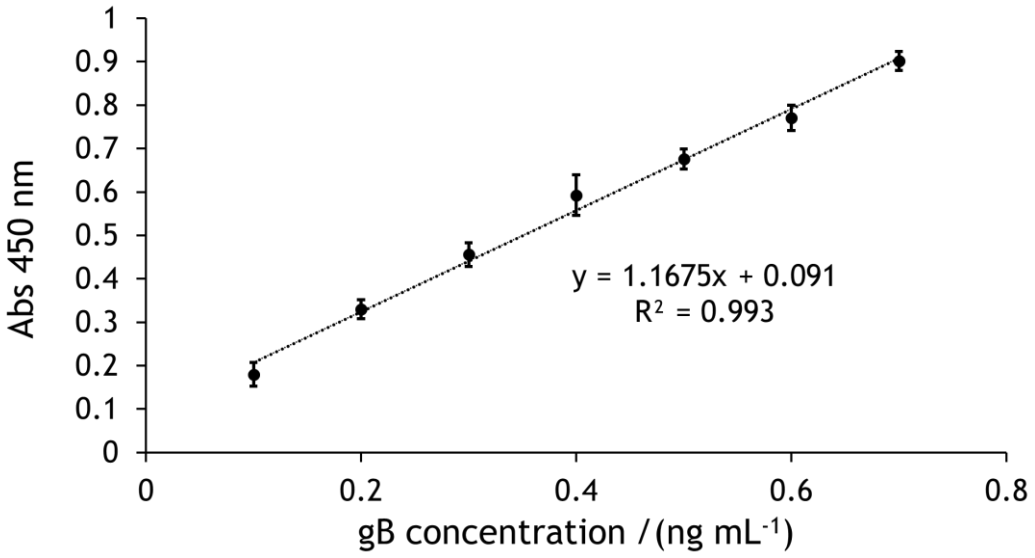

Figure III. 6. Calibration curve for mpEIA in urine diluted 1:2 (v/v) in PBS buffer from 0.09 to 0.70 ng mL^{-1}. Experimental conditions: 5 µg of saturated with antibody MBs added to the wells (8 µg of mAb1 per mg of MBs-PrG). Results are the mean±SD of n=3 replicates.

In both calibration curves, three replicates for each gB concentration were performed and the RSD exhibits an average value of 13.7% when PBS matrix is used and 6.25% in urine samples. These RSD values are acceptable for this kind of immunosensors and the very low concentrations of gB measured, at the pg mL^{-1} order [26].

The lowest detection (LOD) and quantification (LOQ) limits are one of the most important properties in applying immunosensors to clinical samples and to obtain the least number of false negatives. The first stage in LOD calculation was the determination of a linear relation between gB concentration and absorbance signal at 450 nm (Table III.1.). Anomalous points were avoided using least median squares regression (LMS) [27,28]. Then, in order to check the LOD values, results from the calibration curves were evaluated by using the DETARCHI software [29], that calculates detection limits with an evaluation of the probability of false positive (α) and negative (ß), according to ISO11843-2, 2000 [30]. A limit of detection of 70±10 pg mL^{-1} for gB in buffer and 90±20 pg mL^{-1} for gB in urine samples were obtained for α=ß=0.05.

These two values are very similar, reinforcing the non-existence of interferences from the matrix in the immunosensor response.

Table III. 1. Calibration linear parameters for gB determination in buffer and urine matrix samples. Urine samples were diluted 1:2 (v/v)) in PBS 0.1 M.

	Slope	**R^2**	**Linear concentration range, ng mL^{-1}**	**Detection limit, ng mL^{-1}**
PBS	0.87 ± 0.02	0.997	0.10 –0.80	0.07 ± 0.01
Urine	1.17 ± 0.04	0.993	0.10 – 0.70	0.09 ±0.02

To our knowledge, very few immunosensors for the direct determination of gB have been reported. A piezoelectric affinity sensor has been proposed, which is based on the immobilization of anti gB antibodies on gold electrodes [31]. The sensitivity of this immunosensor is, however, very low (LOD about 1 μg mL^{-1} of gB), owing to the intrinsic low sensitivity of this analytical technique. We have also previously reported an electrochemical immunosensor for gB detection, by using capture anti-gB antibodies absorbed on screen-printed carbon electrodes, and secondary anti-gB antibodies labelled with gold nanoparticles[8]. gB detection was carried out through electrochemical stripping analysis of silver nanoparticles deposited quantitatively on the immunosensor, catalyzed by the nanogold labels [8]. However, the reproducibility of this method (RSDs of about 12%) was not very good owing to the random immobilization of the primary antibody on the working electrode, which resulted in small efficiency of antigen detection, low signals compared to the large amount of this antibody used, and the nonspecific deposition of silver on the sensor surface in this kind of voltammetric detection [32]. In this way, the results obtained with the proposed mpEIA compares favorably to those obtained in other reports of gB detection shown in table III.2, in terms of analytical performance. Of all the methods for the detection of gB, the proposed method is the one that presents lowers limits of detection with shorter sample preparation times (the only need to prepare the urine samples are dilutions with 0.10 M. PBS buffer).

Table III. 2. Comparison of the analytical performance of methods for the detection of HCMV antigens/antibodies.

Method	Biological matrix	Analyte	Concentration range	Limit of detection	Overall analysis time	Reference
ELISA	Blood	IgG	0.4 – 0.6 IU mL^{-1}	---	---	[67]
Biosensor Based on Imaging Ellipsometry	Blood	IgG	0.1-1.0 IU mL^{-1}	0.01 IU mL^{-1}	---	[70]
Quartz crystal microbalance immunosensor	---	gB	2.5 -5 µg mL^{-1}	1 µg mL^{-1}	~20h	[159]
Electrochemical immunosensor	Urine	gB	5-15 ng mL^{-1}	3.2 ± 0.2 ng mL-1	~1h	[8]
Electrochemical Immunoassay	Saliva	pp65	0.1 – 80 ng mL^{-1}	30 pg mL-1	>24h	[161]
mpEIA	Urine	gB	90 - 700 pg mL^{-1}	90 ± 20 pg mL^{-1}	~3 h	This work

The proposed immunochemical method must discriminate the gB analyte of interest from other related viruses in the studied biological samples. The presented approach might have the possibility of false positive results, caused by cross-reactions with some virus of Herpesviridae family, once they are morphologically and structurally related [1,35]. To evaluate the selectivity of the antibody, one virus from each sub-family of Herpesviridae family was selected as possible interfere, namely the Epstein-Barr (EBV) and Varicela-zoster (VZV) viruses. In order to evaluate cross reactivities, concentrations of 300, 400 and 500 pg mL^{-1} of the above virus antigens were assayed, and the gB concentration was measured following the proposed method. The obtained absorbances were compared with a control assay, which consisted in a blank test, i.e. PBS buffer was added instead of the analyte to the immunassay. A false positive was considered when the assay with these viruses produced higher spectrophotometric signals compared to the control assay.

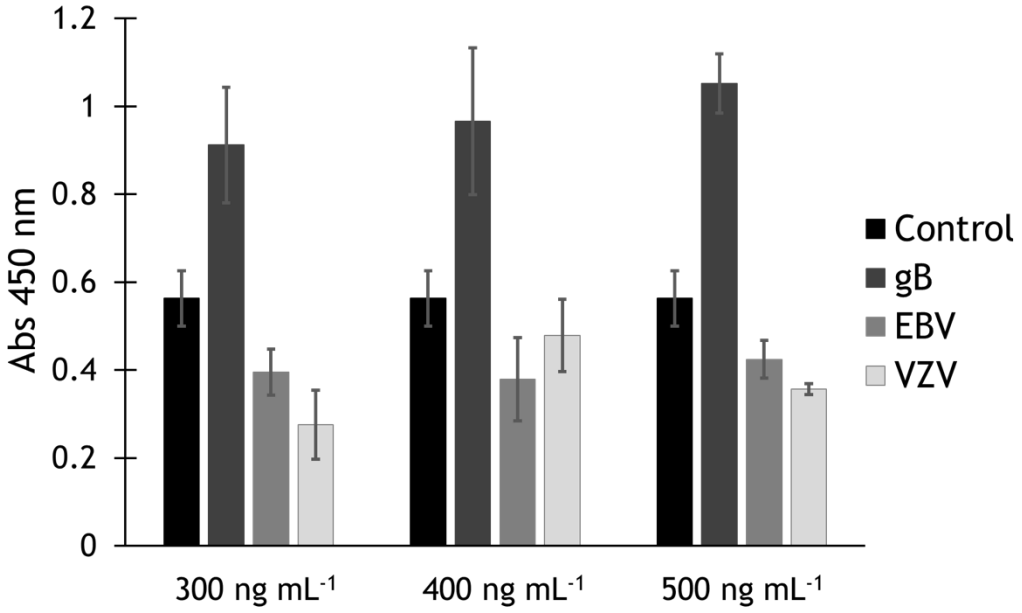

Figure III. 7. Study of the cross-reactivity of EBV and VZV viruses on gB determination, and comparison with control solutions without gB. Results are the mean±SD of n=3 replicates.

According to the results, given in figure III.7., all the assays with EBV and VZV antigens showed lower signals than the control, meaning that the secondary Ab2-HRP did not bind during the assays. In contrast, all the tests with gB showed higher signals, demonstrating the primary and secondary antibody specificities to gB with the sandwich immunoassay scheme.

In order to evaluate the analytical applicability of the proposed mpEIA method, urine samples, collected from a healthy human, were spiked with 300, 400 and 500 pg mL^{-1} of gB and analyzed immediately. The absorbances of the spiked samples, following the mpEIA procedure

(section III.3.4.), were measured and were interpolated in the calibration curve obtained with urine. The results are shown in table III.3. The measurements of each sample were replicated (n=5), and the results of the table are the mean±SD.

Table III. 3. Results of gB determination in spicked urine samples. Calibration equation: $y\,(A) = 1.168\,.\,x\,(ng\,mL^{-1}) + 0.091$. Results are the mean±SD of n=5 replicates.

Spiked gB concentration, ng mL^{-1}	Calculated gB concentration, ng mL^{-1}	% Recovery	Relative error	RSD %
0.30	0.29±0.02	96.6%	3.4%	8.1%
0.40	0.39±0.03	97.5%	2.5%	6.8%
0.50	0.52±0.04	104.0%	4.0%	8.1%

The precision of the mpEIA assay was estimated based on repeat measurements of standard diluted urine samples with spiked concentrations of gB (table III.3). The relative standard deviations have acceptable values, lesser than about 8.1% in all cases (n=5), in accordance with the usually obtained for these types of immunosensors based on magnetic particles compared with classical ELISAs [14,36]. The relative errors at these very low levels of gB concentrations are also smaller than about 4.0% in all cases, demonstrating the successful applicability of the method in urine biological matrices.

It should also be noted the high sensitivity of the developed mpEIA method, being able to detect gB concentrations of few pg mL^{-1}, and a limit of detection of 90 pg mL^{-1} of gB, much smaller than others reported for the same analyte (Table III.2.). For example, as it was reported in our previous work with a disposable electrochemical sandwich immunosensor, the limit of detection was 3.2±0.2 ng mL^{-1} for the same glycoprotein [8].

The overall analysis time of samples with the proposed immunosensor is shorter when compared to the usually applied methods of HCMV virus isolation, DNA amplification, or antibody screening [1]. Approximately 24 h using techniques such as antibody detection and shell-vial and ≈6 h for polymerase chain reaction. The mpEIA can analyze a sample within ≈3 h, a little more compared with our previous work (≈1h) owing to the incubations time needed in this sandwich assay, but with a lower limit of detection and better sensitivity, accuracy, and reproducibility (Table III.2.).

Despite, the most common target analyte used for HCMV quantification being virus DNA, is important to emphasize that our method targets the structural protein, gB, present on the HCMV virus envelope. HCMV is released in urine, during active infection, and our method will allow the infection identification for a minimal concentration of 90±20 pg mL^{-1} gB in this specific body fluid. We expect that the viral load will be directly proportional to gB concentration.

Ongoing work is being developed to establish the correlation between gB concentration and viral load, which will be of great interest to monitor patient response to therapy.

III.5. Conclusions

A sensitive mpEIA is proposed for the rapid (about 3 h) determination of gB in urine samples, with the objective of early screening and quick diagnostics of HCMV infections. Magnetic beads functionalized with PrG allow the oriented immobilization of the primary mAb1 antibody by its Fc part, which has more affinity and efficiency to bind to the antigen protein. Incubations in a dispersed mode, due to the magnetic beads, improve kinetics and also the washing and separating steps of the immunoassay compared with a classical ELISA in which the detecting antibodies are immobilized on the solid surface of the wells of a microtiter plate.

The sandwich immunoassay scheme improves selectivity over other human herpes viruses, given that gB is recognized through two epitopes by both capture and secondary antibodies. The secondary antibody labeled with HRP provides a convenient way to simply obtain the spectrophotometric signals.

The developed method was partially validated following the recommendations of the EMEA and allows the determination of gB in urine samples at a relevant range of concentrations from 90 to 700 pg mL^{-1} of gB. The calculated detection limit in urine samples was 90±20 pg mL^{-1}. Reproducibility exhibits an average RSD value of 6.25 %. Spiked of human urine samples at pg mL^{-1} levels provided relative errors lower than approximately 4.0 %, demonstrating a very good accuracy. The presence of other similar herpes viruses (varicella zoster and Epstein Barr) does not interfere.

ELISA readers are frequently available in biochemical laboratories, but additionally, this same mpEIA method can be easily adapted to electrochemical transduction on screen-printed electrodes. Multiplexed electrochemical transduction of mpEIA provides simplicity, sensitivity and portability of the instrument, as we have previously demonstrated with competitive assay schemes in the determination of drugs of abuse and mycotoxins in foods [37].

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Bibliography

1. Yen-Lieberman, B. Diagnosis of Human Cytomegalovirus Disease. *Clin. Microbiol. Newsl.* **22,** 105–109 (2000).

2. Boeckh, M. & Geballe, A. P. Cytomegalovirus : pathogen, paradigm, and puzzle. *Science in medicine*. **121,** 1673–1680 (2011).

3. Ross, S. A., Novak, Z., Pati, S. & Boppana, S. B. Diagnosis of cytomegalovirus infection. *Infect. Disord. Drug Targets* **11,** 466–74 (2011).

4. *Human Cytomegalovirus*. *Human Cytomegalovirus* (Springer Berlin Heidelberg, 2008).

5. Revello, M. G. & Gerna, G. Diagnosis and Management of Human Cytomegalovirus Infection in the Mother, Fetus, and Newborn Infant. *Clin. Microbiol. Rev.* **15,** 680–715 (2002).

6. Liu, Z. *et al.* Urine real-time polymerase chain reaction detection for children virus pneumonia with acute human cytomegalovirus infection. *BMC Infect. Dis.* **14,** 245 (2014).

7. Baalawi, F., Robertson, P. & Rawlinson, W. D. Detection of human cytomegalovirus IgG antibody using automated immunoassay with recombinant antigens gives uniform results to established assays using whole virus antigens. *Pathology* **42,** 578–580 (2010).

8. Pires, F. *et al.* Disposable immunosensor for human cytomegalovirus glycoprotein B detection. *Talanta* **136,** 42–46 (2015).

9. Demmler-Harrison, G. J. Congenital cytomegalovirus: Public health action towards awareness, prevention, and treatment. *J. Clin. Virol.* **46,** 1–5 (2009).

10. Speckner, A., Glykofrydes, D., Ohlin, M. & Mach, M. Antigenic domain 1 of human cytomegalovirus glycoprotein B induces a multitude of different antibodies which, when combined, results in incomplete virus neutralization. *J. Gen. Virol.* **80,** 2183–91 (1999).

11. Halwachs-Baumann, G. *et al.* Human cytomegalovirus load in various body fluids of congenitally infected newborns. *J. Clin. Virol.* **25 ,** S81-7 (2002).

12. Yamamoto, A. Y. *et al.* Is saliva as reliable as urine for detection of cytomegalovirus DNA for neonatal screening of congenital CMV infection? *J. Clin. Virol.* **36,** 228–230 (2006).

13. Cannon, M. J., Schmid, D. S. & Hyde, T. B. Review of cytomegalovirus seroprevalence and demographic characteristics associated with. *Rev. Med.* 202–213 (2010).

14. Vidal, J. C. *et al.* Rapid determination of recent cocaine use with magnetic particles-based enzyme immunoassays in serum, saliva, and urine fluids. *J. Pharm. Biomed. Anal.* **125,** 54–61 (2016).

15. Park, H., Hwang, M. P. & Lee, K. H. Immunomagnetic nanoparticle-based assays for detection of biomarkers. *Int. J. Nanomedicine* **8,** 4543–4552 (2013).

16. Centi, S., Stoica, A. I., Laschi, S. & Mascini, M. Development of an electrochemical immunoassay based on the use of an eight-electrodes screen-printed array coupled with magnetic beads for the detection of antimicrobial sulfonamides in honey. *Electroanalysis* **22,** 1881–1888 (2010).

17. Makaraviciute, A. & Ramanaviciene, A. Site-directed antibody immobilization techniques for immunosensors. *Biosens. Bioelectron.* **50,** 460–471 (2013).

18. Pei, X. *et al.* Sandwich-type immunosensors and immunoassays exploiting nanostructure labels: A review. *Anal. Chim. Acta* **758,** 1–18 (2013).

19. Ogi, H. *et al.* Nonspecific-adsorption behavior of polyethylenglycol and bovine serum albumin studied by 55-MHz wireless-electrodeless quartz crystal microbalance. *Biosens. Bioelectron.* **24,** 3148–3152 (2009).

20. Guideline on bioanalytical method validation by European Medicines Agency. (2014).

21. Choi, S. & Chae, J. Methods of reducing non-specific adsorption in microfluidic biosensors. *J. Micromechanics Microengineering* **20,** 75015 (2010).

22. Trilling, A. K., Beekwilder, J. & Zuilhof, H. Antibody orientation on biosensor surfaces: a minireview. *Analyst* **138,** 1619–1627 (2013).

23. Steinitz, M. Quantitation of the Blocking Effect of Tween 20 and Bovine Serum Albumin in ELISA Microwells. *Anal. Biochem.* **282,** 232–238 (2000).

24. Zhou, H. *et al.* The sandwich-type electrochemiluminescence immunosensor for α-fetoprotein based on enrichment by Fe3O4-Au magnetic nano probes and signal amplification by CdS-Au composite nanoparticles labeled anti-AFP. *Anal. Chim. Acta* **746,** 107–113 (2012).

25. Rocha-Santos, T. A. P. Sensors and biosensors based on magnetic nanoparticles. *TrAC - Trends Anal. Chem.* **62,** 28–36 (2014).

26. Bertok, T. *et al*. Label-free detection of glycoproteins by the lectin biosensor down to attomolar level using gold nanoparticles. *Talanta* **108,** 11–18 (2013).

27. Massart, D. L. & Kaufman, L. Least median of squares: a robust method for outlier and model error detection in regression and calibration. *Anal. Chim. Acta* **187,** 171–179 (1986).

28. Rousseeuw, P. J. & Leroy, A. M. Robust Regression and Outlier Detection. *J. Appl. Econom.* **4,** 309–311 (1989).

29. Sarabia, L. & Ortiz, M. C. DETARCHI: A program for detection limits with specified assurance probabilities and characteristic curves of detection. *Trends Anal. Chem.* **13,** 1–6 (1994).

30. ISO 11843-2: Capability of detection - Methodology in the linear calibration case. (2000).

31. Susmel, S., O'Sullivan, C. & Guilbault, G. Human cytomegalovirus detection by a quartz crystal microbalance immunosensor. *Enzyme Microb. Technol.* **27,** 639–645 (2000).

32. Escosura-Muñiz, A., Maltez-da Costa, M. & Merkoçi, A. Controlling the electrochemical deposition of silver onto gold nanoparticles: Reducing interferences and increasing the sensitivity of magnetoimmuno assays. *Biosens. Bioelectron.* **24,** 2475–2482 (2009).

33. Sun, H. *et al*. Detection of cytomegalovirus antibodies using a biosensor based on imaging ellipsometry. *PLoS One* **10,** 1–12 (2015).

34. Huang, W. *et al*. Electrochemical Immunoassay for *Cytomegalovirus* Antigen Detection with Multiple Signal Amplification Using HRP and Pt-Pd Nanoparticles Functionalized Single-walled Carbon Nanohorns. *Electroanalysis* **28,** 1126–1133 (2016).

35. Daiminger, A., Bäder, U., Eggers, U., Lazzarotto, T. & Enders, G. Evaluation of two novel enzyme immunoassays using recombinant antigens to detect cytomegalovirus-specific immunoglobulin M in sera from pregnant women. *J. Clin. Virol* **13,** 161–171 (1999).

36. Atanasova, M. Determination of Aflatoxin M1 in Milk by a Magnetic Nanoparticle Based Fluorescent Immunoassay. *Anal. Lett. **5**, 452-469.* (2017).

37. Vidal, J. C. *et al*. A Multi-electrochemical Competitive Immunosensor for Sensitive Cocaine Determination in Biological Samples. *Electroanalysis* **28,** 685–694 (2016).

CHAPTER IV

Magnetic particles-based amperometric immunosensor for Human Cytomegalovirus glycoprotein B detection

The content of this chapter has been submitted to Talanta

Magnetic particles-based amperometric immunosensor for Human Cytomegalovirus glycoprotein B detection

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Keywords: Magnetic beads, immunoassay, mpEIA, Human cytomegalovirus, Glycoprotein B

IV.1. Summary

Nowadays, available methodologies to detect/quantify human cytomegalovirus (HCMV) are cumbersome and/or expensive to be applied routinely as point-of-care. Here, we propose a magnetic particles-based amperometric immunosensor grounded on screen-printed electrodes, for the rapid and sensitive detection of glycoprotein B (gB) in urine samples. Anti-gB antibodies were immobilized in an oriented way onto protein-G functionalized magnetic beads. The approach is based on a sandwich-type immunoassay scheme with gB (HCMV dominant antigen) sandwiched between the primary monoclonal antibody (MBs-PrG-mAb1) and a secondary anti-gB antibody labelled with *Horseradish peroxidase* (Ab2-HRP) to allow electrochemical detection.

The European Medicines Agency (EMEA-2014) guidelines, were used to validate the proposed electrochemical biosensor. Calibration curve performance, matrix effect, lower limit of detection, precision, selectivity and accuracy were assessed in order to ensure the acceptability and validation of the proposed bioanalytical method. A linear dependence between gB concentration and the current intensity in a range of concentrations from 0 to 40 ng mL^{-1} was observed, resulting in a detection limit of 60 ± 10 pg mL^{-1}. RSD was about 8.1 % indicating a good reproducibility. No cross-reactivity was observed with other viruses from the *Herpesviridae* family, namely varicella zoster and Epstein Bar*r* viruses and recovery percentages from spiked human urine samples ranged from about 97 % to 117 %, showing the method accuracy.

On balance, the developed biosensor was shown to allow rapid, successful and easy quantification of gB in urine samples, being capable to be used as point-of-care.

IV.2. Introduction

Human Cytomegalovirus (HCMV) is a herpesvirus which establish a lifelong latent infection that can suffer reactivation time to time [1-6]. HCMV infection can cause serious problems or even be fatal in immunosuppressed individuals, or those with immature immune system [1-6], so its diagnosis is very important. One of the obstacles to carry out such a screening, lays on the analytic means currently offered. Nowadays, the standard methods for HCMV diagnosis are based on PCR [7] , serological tests [8] and viral isolation in tissue, urine or saliva samples [9]. However, all of these methods are expensive or time-consuming, making them disadvantageous to be routinely used as point-of-care tests in clinical diagnosis [10]. Thus, our

group has been working on an alternative that may overcome the limitations presented by the up mentioned standard methods.

Immunoassays, with targeted antigen-antibody complexation, using electrochemical detection coupled to system miniaturization obey the requirements for routine use.

In this sense, we focus the development of our biosensor on a sandwich-type immunoassay by using antibodies against HCMV glycoprotein (the major antigen on the HCMV envelope), so gB protein could be accurately detected in body fluids, like urine and saliva, where viral loads are known to be higher [10,11].

Electrochemical detection was used due to its known enhanced sensitivity and reduced instrumentation costs compared to other transducing methods. Also, to develop miniaturized, manageable, reliable disposable electrochemical devices, screen-printed technology is a clever choice, once it allows massive achievement of electrodic systems with uniform size and geometry, ensuring measurement reproducibility at small cost [12].

Screen-printed electrodes (SPEs) are a well-established technique for the fabrication of electrochemical miniaturized sensors with high sensitivity and selectivity [13,14]. Based on this technology, we have previously reported an immunosensor for gB detection, by using capture anti-gB-HCMV antibodies absorbed on screen-printed carbon electrodes, and a secondary anti-gB-HCMV antibody labelled with gold nanoparticles [10]. gB detection was carried out over electrochemical stripping analysis of silver nanoparticles quantitatively deposited on the immunosensor through catalysis by nanogold labels [10]. Still, the reproducibility of the method (RSDs of about 12%) was not very good owing to the random immobilization of the primary antibody on the working electrode, which resulted in small efficiency of antigen detection (low signals were observed considering the large amount of antibody used). Contributing to the low observed RSD was also the nonspecific deposition of silver on the sensor surface, common to this kind of voltammetric detection [15]. For these reasons, we decided to develop another approach to overcome the observed limitations.

We developed a spectrophotometric magnetic particle-based enzyme immunoassays (mpEIA) [11]. The use of magnetic beads (MBs) functionalized with protein G (MBs-prG) as solid surface for primary antibody (mAb1) immobilization allows its oriented attachment, resulting in a more effective recognition of gB [16]. Additionally, they improve the affinity interaction thanks to a faster assay kinetics of the dispersed beads in urine samples [17,18]. The results obtained with this spectrophotometric mpEIA compared favorably to those obtained in other reports of gB detection in terms of analytical performance [11]. Nevertheless, despite the advantages, ELISA readers cannot be applied as portable devices to make *in situ* measurements.

To overcome this limitation, here we propose the adaptation of the up mentioned mpEIA method to electrochemical transduction on screen-printed electrodes. This variation aims the achievement of a simple, sensitive, disposable and portable device. It is maintained the immunoassay scheme based on the analyte protein gB sandwiched between a primary monoclonal antibody and a secondary anti-gB labelled antibody. Similarly, magnetic particles functionalized with protein G (MBs-prG), are used to allow the antibody (mAb1) oriented

immobilization [10,11,19,20]. And the previously used secondary antibody labelled with *Horseradish peroxidase* (Ab2-HRP), besides allowing spectrophotometric detection, also allows electrochemical detection.

The developed immunosensor was shown to be a fast and effective method of detecting gB in human urine samples for the valuable diagnosis of HCMV infections.

IV.3. Material and methods

IV.3.1. Apparatus

Autolab PGSTAT128N electrochemical system with the General Purpose Electrochemical System (GPES) software version 4.9 (Echo Chemie, Utrecht, Netherlands) was used to perform the electrochemical measurements. Sarstedt (Nümbrecht, Germany) supplied the standard 96-well polystyrene microplates (ref. 82.1581). In order to make easier the removal of solvent from the standard ELISA plates, a magnetic 96-well separator (ThermoFisher Scientific, Waltham, Massachusetts, USA) was used to separate the MBs from the supernatants. The incubation process was carried out using the Grant Bio POS-300 Orbital Shaking Platform (Cambridge, UK). The magnetic separation stand for eppendorf vials (Z5342, 12 positions, 1.5 mL volume) was purchased from Promega (Madison, Wisconsin USA). And a Hanna instruments HI 221 pH meter (USA) was used to measure solutions pH.

IV.3.2. Reagents

Abcam (Cambridge, UK) supplied the human cytomegalovirus glycoprotein B (gB) and Anti-Cytomegalovirus glycoprotein B primary and secondary antibodies (mAb1 and Ab2-HRP) (ab43040, ab6499, ab69245, respectively). Bovine serum albumin (BSA), peroxidase substrate 3,3',5,5'-tetramethylbenzidine liquid substrate system for ELISA (TMB) and Monosodium phosphate were obtained from Sigma-Aldrich (Madrid, Spain). Disodium phosphate was acquired from Panreac (Barcelona, Spain). Sodium chloride was purchased from Fisher Scientific, (Bishop, UK) and dynabeads® protein G immunoprecipitation kit and Pierce Protein-Free were products from Thermofisher Scientific (Waltham, Massachussetts, USA). In the fabrication of screen-printed electrodes several inks were used, namely Electrodag PF-407 A (carbon ink), Electrodag 6037 SS (silver/silver chloride ink), Electrodag 418 (silver ink) and Electrodag 452 SS (dielectric ink) supplied by Achenson Colloiden (Scheemda, Netherlands). The SPEs, composed

by carbon auxiliary and working electrodes and a Ag/AgCl pseudo-reference electrode, were produced on a DEK 248 printing machine (DEK, Weymouth, UK), according to the description in reference [10]. Urine samples were obtained from healthy volunteers after informed consent. All the reagents used, were of analytical grade and Milli Q water (Millipore, Bedford, USA) was used for preparing all solutions.

IV.3.3. Primary antibody immobilization on functionalized magnetic beads

The detailed procedure for primary antibody immobilization on functionalized magnetic beads has been described in an earlier publication [11]. Briefly, dynabeads® were re-suspended in a vial and cleaned. Next, were incubated with the desired amount of primary antibody (mAb1) and washed. Finally, the antibody modified MBs (MB-PrG-mAb1) were reconstituted in PBS and stored at 4 ºC until use.

IV.3.4. Immunoassay determination of gB

All the immunoassay incubations were made according to the procedure described in reference [11], with only minor adjustments. gB standards (or the clinical samples) were added to obtain a final concentration over a range from 0 to 40 ng mL^{-1} gB and secondary antibody (Ab2-HRP) incubation time was reduced to 20 minutes. Also, in the present study to test the immunoassay performance when the biological matrix was used, gB standards were prepared in a 2-fold diluted urine sample from healthy patients in PBS buffer pH 7.4. For the electrochemical measurements, 10 µL of the solution containing the immunoreaction (and also containing Ab2-HRP) were deposited on the screen-printed electrode surface, then, 150 µL of the TMB substrate system reagent was added to the electrode surface, left to react for 15 min at room temperature and determined by amperometric detection at a constant potential of -0.2 V (vs. Ag pseudo-reference electrode) at a fixed time of 90 s. The sensor electrodes were simply washed with PBS buffer between electrochemical measurements.

IV.4. Results and Discussion

A schematic representation of the magnetic-based amperometric immunosensor for the determination of gB in urine samples is shown in scheme IV.1. As mentioned, the proposed approach is based on a sandwich-type immunoassay, with the HCMV glycoprotein B sandwiched between the MBs-PrG-mAb1 and Ab2-HRP. In a final step, the modified MBs are transferred to the working electrode on the SPEs for electrochemical transduction. The amount of peroxidase enzyme (HRP) is quantified by amperometry after adding the TMB liquid substrate system.

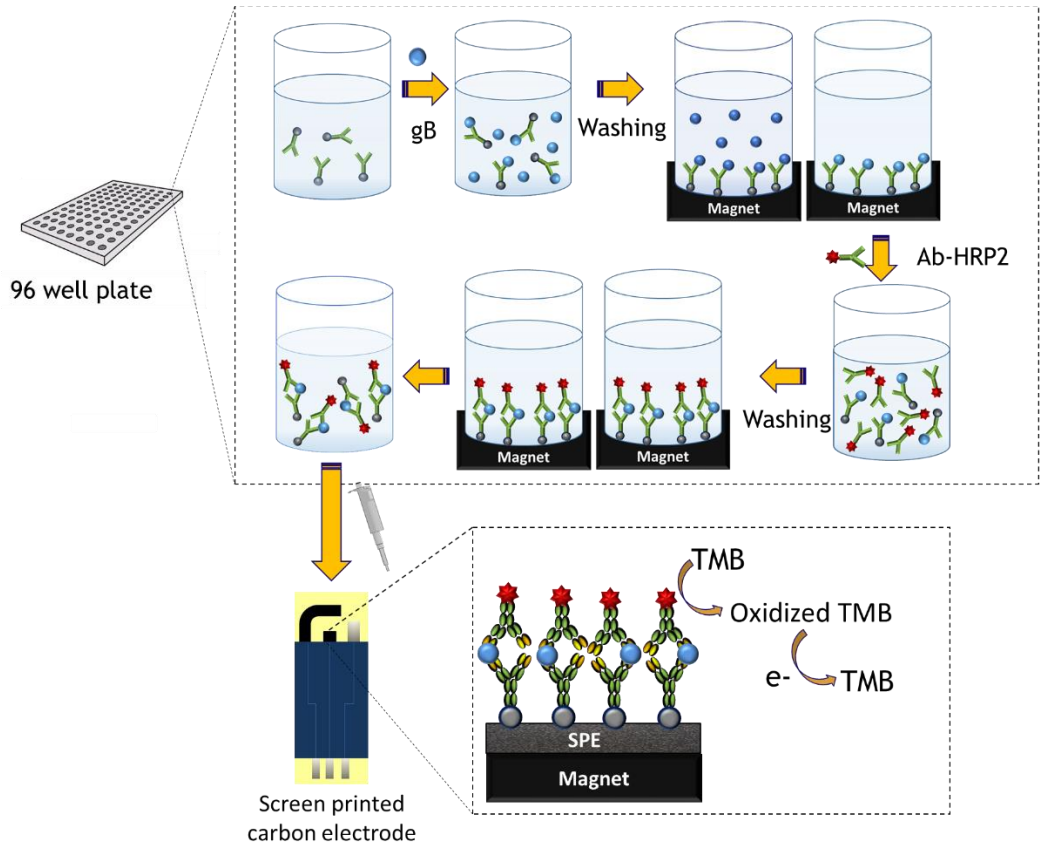

Figure IV. 1. Schematic representation of the developed mpEIA-based electrochemical immunosensor for the sensitive gB quantification.

Immunoassays are greatly dependent on experimental conditions, as consequence, their detailed optimization for achieving the best analytical properties is of great importance. Therefore, several experimental parameters were optimized, such as enzymatic reaction time, antibody-antigen incubation time and temperature. Different sensor surface modifications were also tested, as well as the influence of MBs-PrG and Ab2-HRP concentrations along with the gB quantities on the sensor surface.

Finally, under optimal operation conditions, the immunosensor performance was tested and validated according to the "Guideline on bioanalytical method validation", from the European Medicines Agency (EMEA) (2014) by electrochemical determination of gB in human urine samples. Evaluation of cross-reactivity with Epstein-Barr virus (EBV) and Varicela-zoster (VZV) antigens, both viruses from the *Herpesviridae* family was also performed [21].

IV.4.1. Parameters optimization

In order to obtain a signal with improved sensitivity some modification on the sensor surface were performed. Gold nanoparticles (AuNPs) present good biocompatibility, increase the surface area and facilitate electrons transfer between redox proteins and the electrode surface [22-24]. On the other hand, carbon materials, such as carbon nanotubes (CNTs) are also widely used to functionalize SPEs due to their physical properties, such as large surface area, chemical and thermal stability and electronic properties [25]. In this way, combining SPEs with these materials can improve sensor performance. The signals obtained, while working with an unmodified electrode, were compared with the signals resulting from the electrodes modified with AuNP, CNT and CNT with AuNP (figure IV.2). Signal amplification is observed when the surface electrode was modified with electrodeposited AuNP. None of the other modifications resulted in an improved signal compared to the use of a bare electrode.

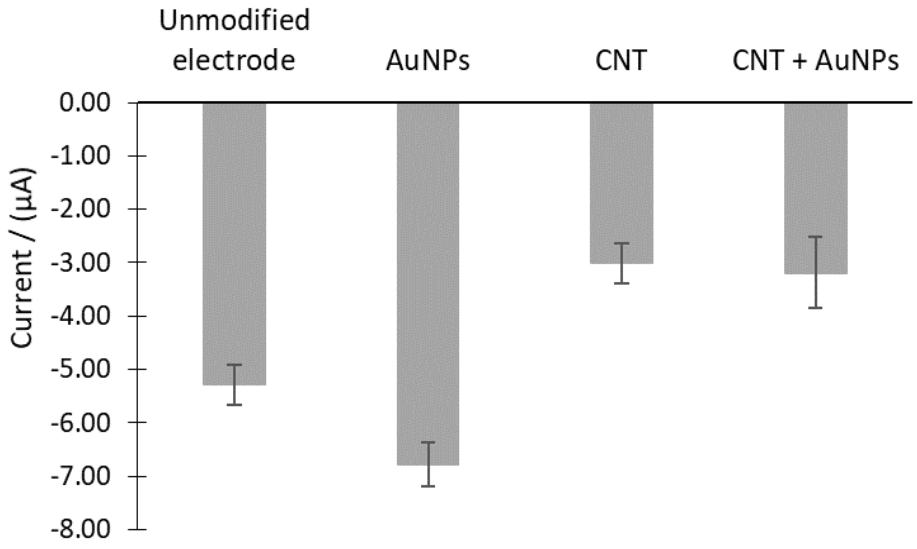

Figure IV. 2. Comparison of response intensities obtained by using different modifications onto the electrode surface. An excess concentration of 1 µg mL^{-1} gB was used. Results are the mean±SD of n=5 measurements.

Common ELISA procedures recommend incubating TMB for 15-30 min. Nevertheless, with the purpose to obtain a maximum signal at a shorter time, the required time to let the enzymatic reaction occurs was evaluated. Figure IV.3 shows that at the initial reaction instants we always obtain a higher signal, however, when comparing this current response with the ones obtained at the other analyzed incubation periods, a deformation of the signal is generally observed (data not shown). After 15 min the current response remains constant, making in interest of time, 15 min the chosen interval for the enzymatic reaction.

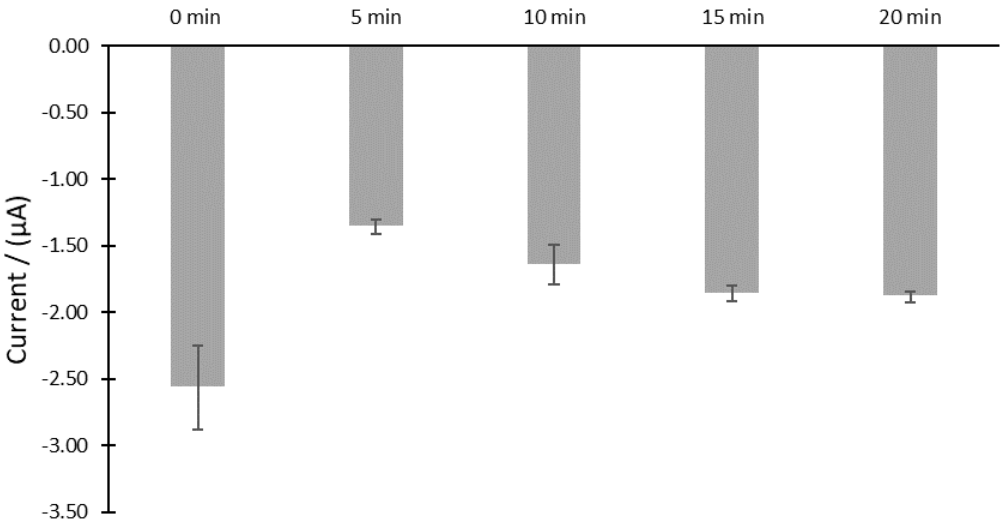

Figure IV. 3. Influence of the enzymatic reaction incubation time on TMB_{ox} reduction signal. An excess concentration of 1 µg mL^{-1} gB was used. Results are the mean ±SD of n=5 measurements.

Magnetic particles based immunobindings take advantage of immobilization of the antibodies on individual micro-particles rather than in a solid surface, where antigen access is more difficult and greater problems of nonspecific adsorptions can occur. Also a magnetic separator allows fast and simple removal of the supernatants during washing and separation steps with great efficiency [17]. Yet, the total amount of MBs needs to be tuned, as it influences the amount of antibody binding sites [26].

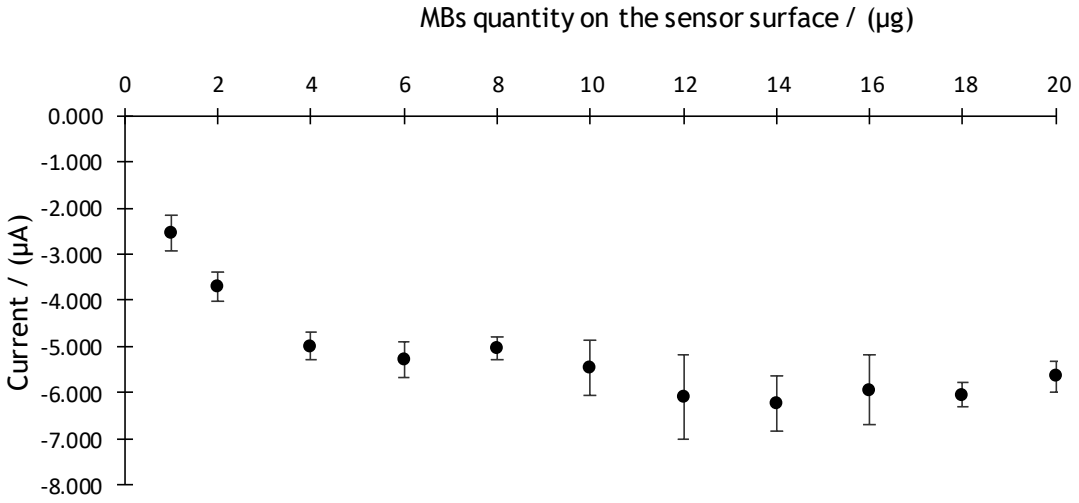

Figure IV. 4. Optimization of the amount of MBs-PrG-mAb1 to be used on the sensor surface. An excess concentration of 1 µg mL^{-1} gB was used. Results are the mean±SD of n=5 measurements.

With the aim to guarantee the best electrochemical signal, the current response was evaluated by varying, on the sensor surface, the amount of modified MBs-PrG in presence of mAb1 excess. Results (figure IV.4) indicate that for quantities higher than 6 µg of MBs-PrG-mAb1 on the sensor surface (30 µg in the well) the reduction current response tends to be constant. Being this amount of MBs-PrG in excess of mAb1 considered the optimal one, as it gives a good current response with low dispersion for the minimum quantity of MBs-PrG-mAb1.

Another important parameter to be optimized in this type of immunoassay is the reaction temperature. As antibodies and enzymes are proteins, optimal response of the immunoassay is expected when operated under physiological conditions (37 °C for temperature). A comparison of the immunoassay response under temperatures of 4°C, 37°C and room temperature was made. The obtained results are shown in figure IV.5.

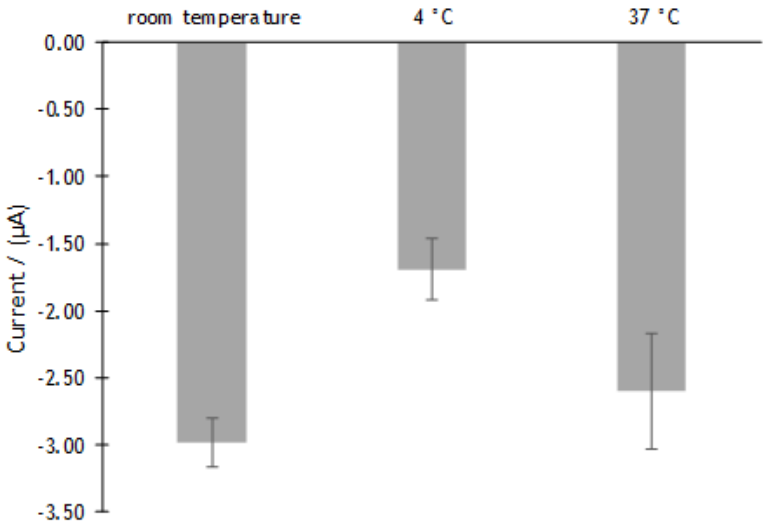

Figure IV. 5. Temperature effect on the immunoassay response. An excess concentration of 1 µg mL^{-1} gB was used. Results are the mean±SD of n=5 measurements.

As expected, working at room temperature and at 37°C gave higher signals when compared to 4°C. However, at 37 ° C a great dispersion was observed. Thus, it was stipulated that room temperature is the best alternative for this immunoassay.

The antigen-antibody incubation time also must be optimized because it allows to reduce significantly the analysis time. Thus, the effect of this incubation time on the signal reduction was evaluated. According to figure IV.6., it can be said that in general the signal has a constant intensity, only at 10 minutes of incubation a slightly lower signal is observed when compared to other incubation times. In this way, 20 minutes was chosen as the optimal time for incubation once is the shorter time with the observed lowest dispersion.

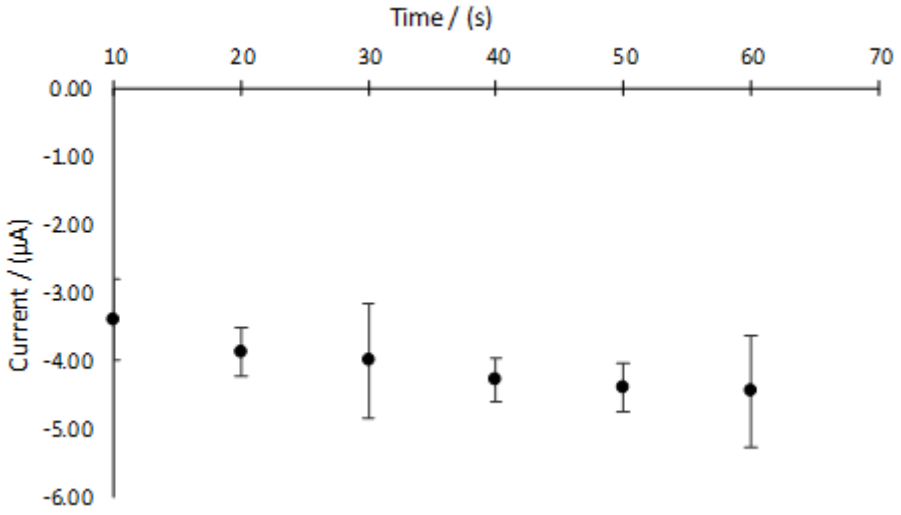

Figure IV. 6. Effect of antigen-antibody incubation time on current signal. An excess concentration of 1 µg mL^{-1} gB was used. Results are the mean±SD of n=5 measurements.

Antibodies are one of the most expensive compounds of this immunoassay, so, to reduce costs, an optimization in order to use the minimum amount of secondary antibody (Ab2-HRP), without affecting the signal response was performed. Concentration of 0.050, 0.125, 0.250, 0.500, 0.750, 1 µg mL^{-1} were tested and the results are presented in figure IV.7. From 0.050 to 0.500 µg mL^{-1} of Ab2-HRP, the signal intensity increases with the secondary antibody concentration. After this, the current tends to be constant. Thus, 0.500 µg mL^{-1} was chosen as the optimal Ab2-HRP concentration to be used on this electrochemical immunoassay procedure.

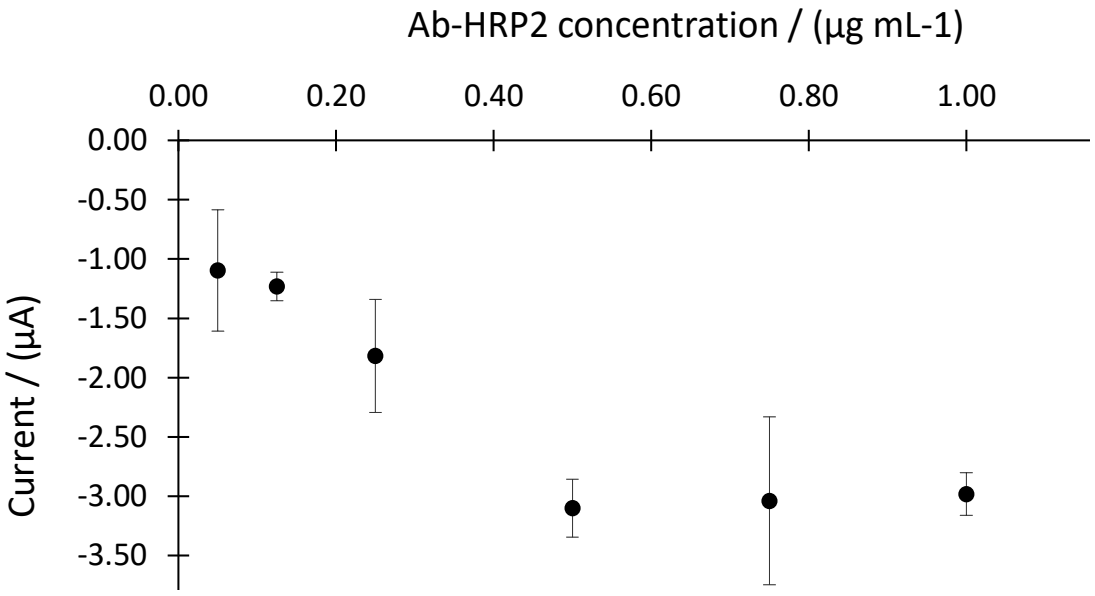

Figure IV. 7. Effect of Ab2-HRP concentration on current signal. An excess concentration of 1 µg mL^{-1} gB was used. Results are the mean±SD of n=5 measurements.

All the optimized experimental conditions are summarized on "Materials and Methods" sections 2.3 and 2.4 and in Table IV.1. The optimized amperometric transduction conditions (substrate concentrations and amperometric parameters) were previously optimized in [27].

Table IV. 1. Summary of the optimal parameters of mpEIA-based sandwich electrochemical immunosensor

mpEIA-based sandwich electrochemical immunosensor	
Blocking the wells	300 µL of Pierce Protein-Free, overnight
Blocking of the remaining sites of the beads surface	100 µL of BSA 4%, 60 minutes
Modifications onto the electrode surface	Electrodeposition of AuNP
Quantity of MBs-PrG-mAb1 on sensor surface.	6 µg of MBs-PrG-mAb1 (30 µg in the well)
immunoreaction temperature	Room temperature
Antigen-antibody incubation time	20 minutes
Ab2-HRP concentration	0.500 µg mL-1

Table IV.1. Summary of the optimal parameters of mpEIA-based sandwich electrochemical immunosensor (continued).

Electrochemical Transduction conditions	
Volume transferred for sensor surface	10 µL of the immunoreaction
Enzymatic substrate	TMB
Enzymatic reaction incubation time	15 minutes
Amperometric detection	-0.2 V (vs Ag/AgCl), 90s

IV.4.2. Immunosensor Validation

As in the previous developed mpEIA [11], the guidelines of the European Medicines Agency on bioanalytical method validation were used to evaluate the immunoassay analytical performance [21]. Thus, calibration curve performance, matrix effect, lower limit of detection, precision, selectivity and accuracy were assessed in order to ensure the acceptability and validation of the proposed bioanalytical method.

For comparison purposes with the previously developed mpEIA [11], calibration standards were prepared in PBS buffer and urine matrices. High virus load is found in excreted urine from HCMV infected individuals, thus this biological sample is used to evaluate the immunosensor performance [28,29]. For both matrices (PBS and urine), current increased linearly with analyte concentration (figures IV.8 b) and IV.9) respectively in the range from 0 ng mL^{-1} to 60 ng mL^{-1} of gB (figure IV.8 a)) and in the range from 0 ng mL^{-1} to 40 ng mL^{-1} of gB (figure IV.9). As can be seen from table IV.2, calibration curves show good linear correlation coefficients. These results compare well with the ones obtained by the previously developed mpEIA [11], providing as well, clinically useful information for HCMV quantification at low concentrations. Limits of detection (LOD) of 0.07±0.01 ng mL^{-1} for gB-HCMV in buffer and 0.06±0.01 ng mL^{-1} for gB in urine samples were obtained for α=β=0.05. For these values attainment, calibration curves were evaluated using the DETARCHI software [30], which calculates detection limits with an evaluation of the probability of false positive (α) and false negative (β), according to ISO11843-2, 2000 [31]. The similarity between the LOD values obtained in presence of PBS and urine (table IV.2), once more, reinforces the non-existence of interferences from the matrix on the immunosensor response. Indeed, this is to be expected, since the used magnetic-particles based immunoassay has the advantage of excluding matrix influence on the electrochemical detection. The bead-based immunobinding captures the gB biomolecule out of the matrix. Due

to the transfer of the beads in PBS buffer solution to the electrode, the matrix is excluded from the amperometric measurement.

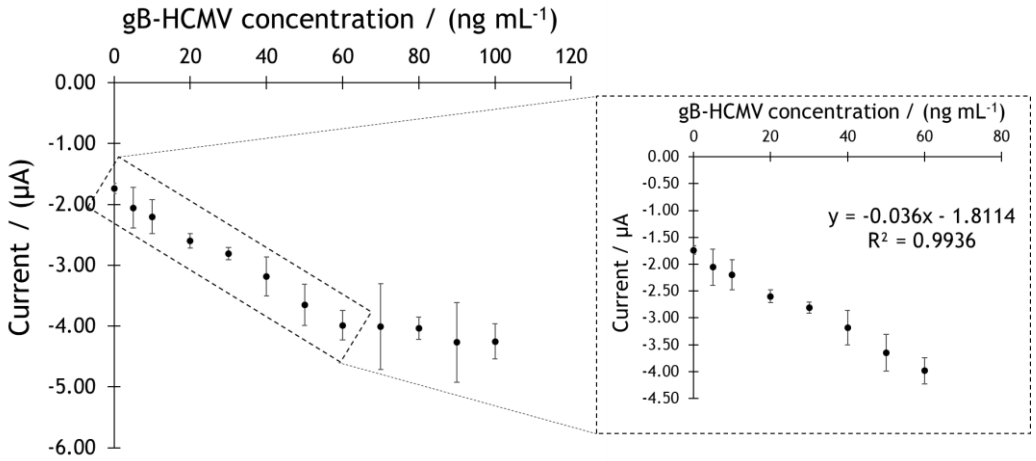

Figure IV. 8. a) Immunosensor current response for gB concentrations from 0 to 100 ng mL^{-1} in 0.1 M. buffer matrix. b) calibration curve from 0 to 60 ng mL^{-1}. Experimental conditions: 6 µg of MBs-PrG-mAb1 added to the sensor surface (8 µg of mAb1 per mg of MBs-PrG). Results are the mean±SD of n=3 measurements.

Table IV. 2. Calibration linear parameters for gB determination in buffer and urine matrix samples. Urine samples were diluted 1:2 (*v*/*v*)) in PBS 0.1 M.

	Slope	**R^2**	**Linear concentration range, ng mL^{-1}**	**Detection limit, ng mL^{-1}**
PBS	-0.036 ± 0.001	0.994	0 –60	0.07 ± 0.01
Urine	-0.037 ± 0.001	0.997	0 – 40	0.06 ± 0.01

To evaluate the within-run precision, in both calibration curves, three replicates for each gB concentration were carried out and the RSD calculated, which exhibited an average value of 8.4% when PBS matrix was used and 8.1% in urine samples. These results, as in the case of the mpEIA [11], can be considered very good for this kind of immunosensors [32] and the low concentrations of gB measured, at the pg mL^{-1} order [11].

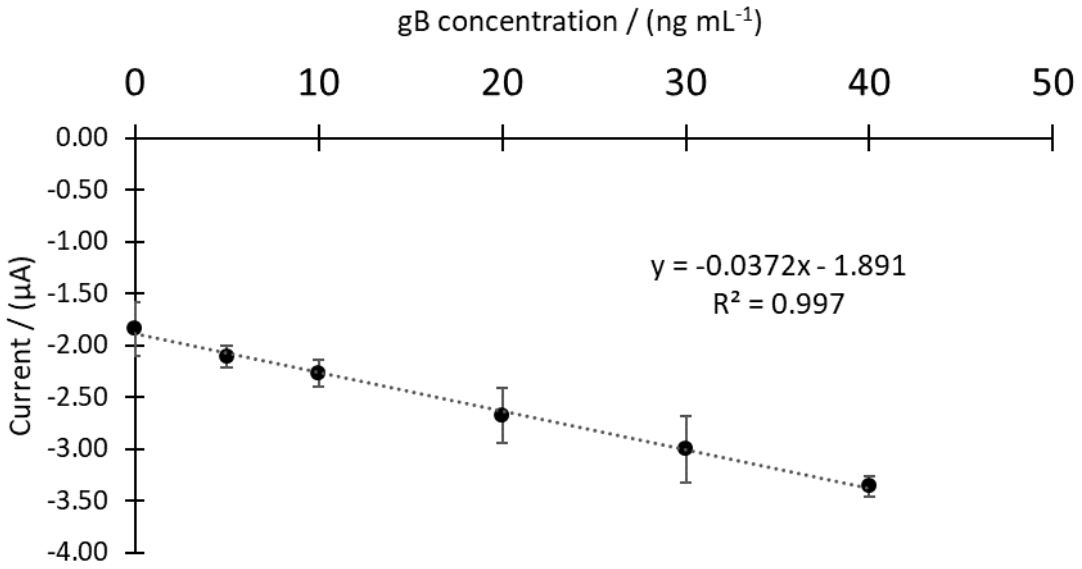

Figure IV. 9. Immunosensor current response for gB concentrations from 0 to 40 ng mL^{-1} in urine matrix (dilute 1:2 in 0.1M buffer pH 7.4). Experimental conditions: 6 µg of MBs-PrG-mAb1 added to the sensor surface (8 µg of mAb1 per mg of MBs-PrG). Results are the mean±SD of n=3 measurements.

The analytical performance of the proposed magnetic particles-based electrochemical immunosensor compares favorably to those obtained in other reports for gB detection [11]. Of all the methods for gB-HCMV detection, the presently proposed is the one that grants the lowest limits of detection with the shorter sample analysis time.

The selectivity of the present developed electrochemical immunosensor was also evaluated. The Epstein-Barr (EBV) and Varicela-zoster (VZV) viruses from each sub-family of *Herpesviridae* family were tested for cross reactivity estimation. Concentrations of 10, 20 and 30 ng mL^{-1} of the above viruses antigens were assayed, and the gB-HCMV concentration measured following the proposed method. The obtained reduction current was compared with a control assay, which consisted in a blank test [163]. Results shown that all the assays with EBV and VZV antigens exhibited lower or similar signals when compared to the control (figure IV.10), meaning that the secondary Ab-HRP2 did not bind during the assay. In contrast, all the tests with gB-HCMV shown higher signals, reinforcing the primary and secondary antibody specificities to gB-HCMV within the proposed sandwich immunoassay scheme.

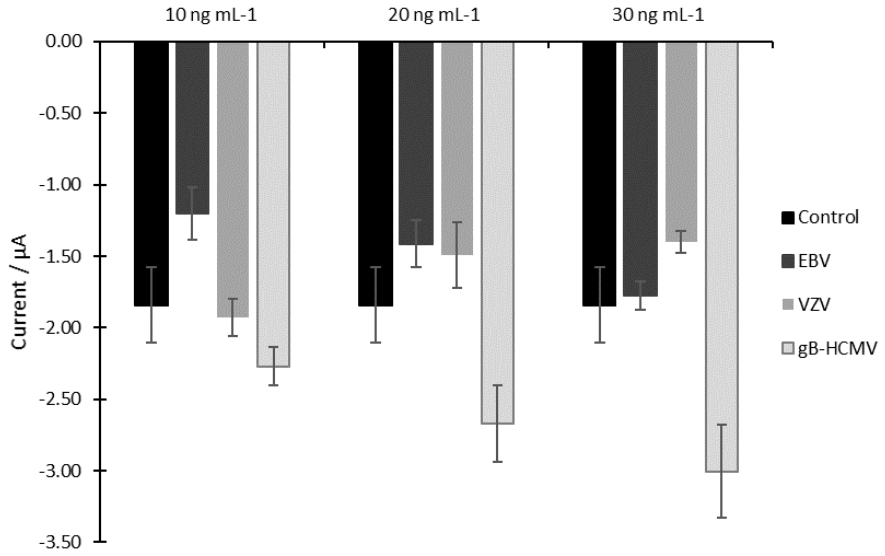

Figure IV. 10. Study of the cross-reactivity of EBV and VZV viruses on gB determination, and comparison with control solutions without gB. Results are the mean±SD of n=3 measurements.

In order to evaluate the accuracy of the proposed immunoassay, urine samples, collected from a healthy human, were spiked with 10, 20 and 30 ng mL^{-1} of gB-HCMV and analyzed immediately to study recovering percentages under the optimized procedures. Each sample was replicated three times (n=3). All the results are summarized in table IV.3. The current obtained from the spiked samples were measured and were interpolated in the calibration curve obtained with urine. The results are shown in Table IV.3. The recovery percentages ranged between about 97 to 117 % and the error percentages at the studied spiked concentration levels of gB had good analytical values ranging between 3 to 17%.

Table IV. 3. Results of gB determination in spicked urine samples. Calibration equation: $y\ (\mu A) = -0.0372\ .x\ (ng\ mL^{-1}) - 1.891$ Results are the mean±SD of n=3 measurements

Spiked gB-HCMV concentration, ng mL^{-1}	Calculated gB-HCMV concentration, ng mL^{-1}	% Recovery	Error %
10	11.71±0.36	117.1%	17.1%
20	20.86±0.42	104.3%	4.3%
30	29.09±0.46	97.0 %	3.0%

Finally, it should also be noted that the developed electrochemical immunosensor is still able to detect gB-HCMV concentrations in the pg mL^{-1} order, but with a lower limit of detection (60 pg mL^{-1} of gB-HCMV) when compared with the previously developed mpEIA [11].

Also the overall samples analysis time was reduced from 3 hours [11] to ≈2 hours.

In summary, the developed method for gB determination allow its sensitive quantification in urine samples at the pg mL^{-1} level with high accuracy and precision and in a reduced time span.

IV.5. Conclusions

A magnetic particles-based electrochemical immunosensor is proposed for the rapid (about 2 h) quantification of gB in urine samples, with the objective of early screening and quick diagnostics of HCMV infections. Magnetic beads functionalized with PrG allow the oriented immobilization of the primary mAb1 antibody by its Fc part, due to its affinity for PrG. The presence of magnetic beads also improves kinetics and the washing and separating steps of the immunoassay.

The sandwich immunoassay scheme improves selectivity over other human herpes viruses, given that gB is recognized through two epitopes by both capture and secondary antibodies. Actually, the presence of other similar herpes viruses (varicella zoster and Epstein Barr) did not induce false positives. Also, the secondary antibody labeled with HRP provides a convenient way to simply obtain the electrochemical signal.

The developed method was partially validated following the recommendations of the EMEA. A linear dependence between gB concentration and the current intensity in a range of concentrations from 0 to 40 ng mL^{-1} was observed in presence of urine samples, resulting in a detection limit of 60 ± 10 pg mL^{-1}. RSD was about 8.1 % indicating a good reproducibility. Lastly, recovery percentages from spiked human urine samples ranged from about 97 % to 117 %, showing the method accuracy.

The analytical performance of the proposed electrochemical immunosensor compares favorably to those obtained in other previous reports for gB-HCMV detection, showing lower limits of detection with shorter sample time analysis. These promising results provide excellent guidelines for device improvement.

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Bibliography

1. Revello, M. G. & Gerna, G. Diagnosis and Management of Human Cytomegalovirus Infection in the Mother, Fetus, and Newborn Infant. *Clin. Microbiol. Rev.* **15**, 680–715 (2002).

2. Sanchez, V., Greis, K. D., Sztul, E. & Britt, W. J. Accumulation of Virion Tegument and Envelope Proteins in a Stable Cytoplasmic Compartment during Human Cytomegalovirus Replication: Characterization of a Potential Site of Virus Assembly Accumulation of Virion Tegument and Envelope Proteins in a Stable C. *J. Virol.* **74**, 975–986 (2000).

3. Yen-Lieberman, B. Diagnosis of Human Cytomegalovirus Disease. **Clin. Microbiol. Newsl. 22**, 105–109 (2000).

4. Boeckh, M. & Geballe, A. P. Cytomegalovirus : pathogen, paradigm, and puzzle. *Science in medicine*. **121**, 1673–1680 (2011).

5. Human Cytomegalovirus. Human Cytomegalovirus (Springer Berlin Heidelberg, 2008).

6. Landolfo, S., Gariglio, M., Gribaudo, G. & Lembo, D. The human cytomegalovirus. *Pharmacol. Ther.* **98**, 269–297 (2003).

7. Liu, Z. et al. Urine real-time polymerase chain reaction detection for children virus pneumonia with acute human cytomegalovirus infection. *BMC Infect. Dis.* **14**, 245 (2014).

8. Baalawi, F., Robertson, P. & Rawlinson, W. D. Detection of human cytomegalovirus IgG antibody using automated immunoassay with recombinant antigens gives uniform results to established assays using whole virus antigens. *Pathology* **42**, 578–580 (2010).

9. Demmler-Harrison, G. J. Congenital cytomegalovirus: Public health action towards awareness, prevention, and treatment. *J. Clin. Virol.* **46S**, 1–5 (2009).

10. Pires, F. et al. Disposable immunosensor for human cytomegalovirus glycoprotein B detection. *Talanta* **136**, 42–46 (2015).

11. Pires, F., Dias-Cabral, A. C., Arcos-Martínez, M. J., Vidal, J. C. & Castillo, J. R. A rapid magnetic particle-based enzyme immunoassay for human cytomegalovirus glycoprotein B quantification. *J. Pharm. Biomed. Anal.* **156**, 372–378 (2018).

12. Renedo, O. D., Alonso-Lomillo, M. A. & Martínez, M. J. A. Recent developments in the field of screen-printed electrodes and their related applications. *Talanta* **73**, 202–219 (2007).

13. Thiyagarajan, N., Chang, J. L., Senthilkumar, K. & Zen, J. M. Disposable electrochemical sensors: A mini review. *Electrochem. commun.* **38**, 86–90 (2014).

14. Taleat, Z., Khoshroo, A. & Mazloum-Ardakani, M. Screen-printed electrodes for biosensing: A review (2008-2013). *Microchim. Acta* **181**, 865–891 (2014).

15. Escosura-Muñiz, A., Maltez-da Costa, M. & Merkoçi, A. Controlling the electrochemical deposition of silver onto gold nanoparticles: Reducing interferences and increasing the sensitivity of magnetoimmuno assays. *Biosens. Bioelectron.* **24**, 2475–2482 (2009).

16. Makaraviciute, A. & Ramanaviciene, A. Site-directed antibody immobilization techniques for immunosensors. *Biosens. Bioelectron.* **50**, 460–471 (2013).

17. Vidal, J. C. *et al.* Rapid determination of recent cocaine use with magnetic particles-based enzyme immunoassays in serum, saliva, and urine fluids. *J. Pharm. Biomed. Anal.* **125**, 54–61 (2016).

18. Centi, S., Stoica, A. I., Laschi, S. & Mascini, M. Development of an electrochemical immunoassay based on the use of an eight-electrodes screen-printed array coupled with magnetic beads for the detection of antimicrobial sulfonamides in honey. *Electroanalysis* **22**, 1881–1888 (2010).

19. Speckner, A., Glykofrydes, D., Ohlin, M. & Mach, M. Antigenic domain 1 of human cytomegalovirus glycoprotein B induces a multitude of different antibodies which, when combined, results in incomplete virus neutralization. J. Gen. Virol. 80, 2183–2191 (1999).

20. Pei, X. *et al.* Sandwich-type immunosensors and immunoassays exploiting nanostructure labels: A review. *Anal. Chim. Acta* **758**, 1–18 (2013).

21. Guideline on bioanalytical method validation by European Medicines Agency. (2014).

22. Molinero-Abad, B., Alonso-Lomillo, M. A., Domínguez-Renedo, O. & Arcos-Martínez, M. J. Amperometric determination of sulfite using screen-printed electrodes modified with metallic nanoparticles. *Microchim. Acta* **180**, 1351–1355 (2013).

23. Pingarrón, J. M., Yáñez-Sedeño, P. & González-Cortés, A. Gold nanoparticle-based electrochemical biosensors. *Electrochim. Acta* **53**, 5848–5866 (2008).

24. Cao, X., Ye, Y. & Liu, S. Gold nanoparticle-based signal amplification for biosensing. *Anal. Biochem.* **417**, 1–16 (2011).

25. Rocha-Santos, T. A. P. Sensors and biosensors based on magnetic nanoparticles. *Trends Anal. Chem.* **62**, 28–36 (2014).

26. Vidal, J. C. *et al.* A Multi-electrochemical Competitive Immunosensor for Sensitive Cocaine Determination in Biological Samples. *Electroanalysis* **28**, 685–694 (2016).

27. Bertolín, J. R. Inmunosensores amperométricos y espectrofotométricos para la determinación de micotoxinas en alimentos y drogas de abuso en muestras biológicas - *Tesis Doctora- Universidad de Zaragozal*. (2015).

28. Halwachs-Baumann, G. et al. Human cytomegalovirus load in various body fluids of congenitally infected newborns. *J. Clin. Virol.* **25**, S81-7 (2002).

29. Yamamoto, A. Y. *et al.* Is saliva as reliable as urine for detection of cytomegalovirus DNA for neonatal screening of congenital CMV infection? J. Clin. Virol. 36, 228–230 (2006).

30. Sarabia, L. & Ortiz, M. C. DETARCHI: A program for detection limits with specified assurance probabilities and characteristic curves of detection. *Trends Anal. Chem.* **13**, 1–6 (1994).

31. ISO 11843-2: Capability of detection - Methodology in the linear calibration case. (2000).

32. Bertok, T. *et al.* Label-free detection of glycoproteins by the lectin biosensor down to attomolar level using gold nanoparticles. *Talanta* **108**, 11–18 (2013).

CHAPTER V

Concluding remarks and future perspectives

V.1.Concluding remarks and future perspectives

Electrochemical biosensors play a significant role in medicine, agriculture, food safety, bioprocessing and environmental and industrial monitoring once they can detect and provide accurate and reliable information about molecules of analytical significance, pathogens and toxic compounds. Traditionally such analyses have been performed in the laboratory and are associated with disproportionate expenses for equipment and highly trained personnel. It seems reasonable therefore that industry and society has pushed towards lower cost solutions which can be used even by the consumer. The use of screen-printed electrodes as transducer opens important expectations in this sense, providing the construction of simple, rapid, portable, cost competitive and reagentless biosensors.

The work developed during this thesis aimed the development of a miniaturized electrochemical immunosensor for the detection of human cytomegalovirus (HCMV) in urine samples that could be used as point of care. For this reason, was established that screen-printed electrodes would be a clever option as transducer and that the use of capture antibodies against the envelope glycoproteins of HCMV, more specifically glycoprotein B (gB) would provide highly specificity and selectivity to this method.

In this way, a miniaturized screen-printed-based electrochemical immunosensor for gB-HCMV detection was presented. The detection approach was based on electrochemical stripping analysis of silver nanoparticles quantitatively deposited on the immunosensor through catalysis by nanogold labels. This study achieved variations in the stripping-current response that directly correlated with the variation in HCMV gB concentration and the calculated detection limit in urine was 3.2 ± 0.2 ng mL^{-1} of HCMV gB. Additionally, we were also able to obtain a result after a short period of time (approximately 1 hour). However, due to the random immobilization of the primary antibody on the working electrode, and also to the nonspecific deposition of silver on the sensor surface, the reproducibility of the method (RSDs of about 12%) was not very good.

For this reason, to overcome the limitations found on this sensor, we advanced to a different approach. We developed a spectrophotometric magnetic particle-based enzyme immunoassays (mpEIA). In this method magnetic beads (MBs) functionalized with protein G (MBs-prG) were used as solid surface for primary antibody immobilization. This allows antibody oriented attachment, resulting in a more effective recognition of gB. Furthermore, MBs induce a faster assay kinetics of the dispersed beads and lead to a more efficient washing separating steps of the immunoassay by the application of a magnetic field. The spectrophotometric detection was performed through a secondary antibody labelled with *Horseradish peroxidase* and once again, a linear dependence between gB concentration and the absorbance signal at 450 nm was obtained, showing that this method also responds to gB concentration. In addition,

both RSD and limit of detection suffered a clear decreased. A detection limit of gB-HCMV 90 ± 2 pg mL^{-1} and a RSD of about 6.7% were attained in urine samples. In this way, we have surpassed the previously mentioned reproducibility problem. Still, despite the advantages of this approach and the simplicity of instrumentation, spectrophotometers cannot be applied as portable devices to make *in situ* measurements.

Thus, we propose the adaptation of the up mentioned mpEIA method to electrochemical transduction on screen-printed electrodes. It was maintained the mpEIA immunoassay scheme, but the immunoreaction was transferred to the surface of a screen-printed electrode. The presence of the secondary antibody labelled with *Horseradish peroxidase* (Ab2-HRP) also allowed electrochemical gB detection. This electrochemical sensor not only responded to gB concentration, permitting gB quantification, but also maintained a small detection limit of 0.06±0.01 ng mL^{-1}. Additionally, the problem associated with reproducibility was reduced, once this method presents a RSD of about 8%.

In this way we can say that these results are promising for the development of a fast and effective method for gB quantification, and consequent HCMV diagnostic in human urine samples. Also, this method can be seen as a sensitive, cheaper and faster alternative for the cumbersome diagnostic method available nowadays.

In a future work, it is important to validate the method by study its clinical applicability with real samples. Unfortunately, this process took longer time than we expected, partly due to the difficulty in obtaining enough samples from unhealthy patients. Moreover, the correlation with other established methods is difficult, since no standard method is available for the gB detection. The currently used techniques are based on DNA amplification, antibodies screening or virus isolation in fibroblasts culture. Nevertheless, it is expected that the viral load will be directly proportional to gB concentration, yet it will be necessary to establish the correlation between these two parameters and its further comparison with other quantification standard methods, like PCR.

Finally, it would be interesting to apply this sensor scheme in the diagnosis of different viral infections.

Appendix

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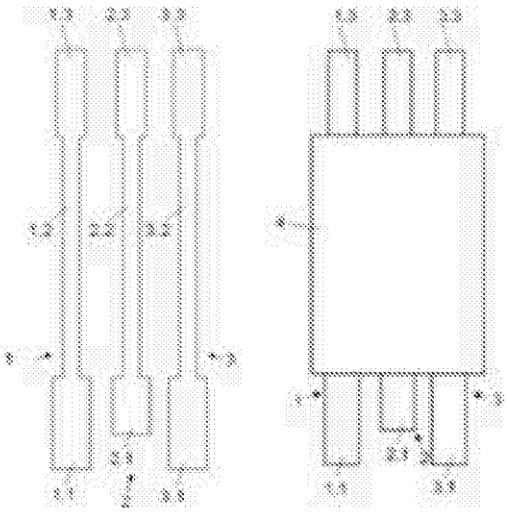
(57) Resumo:



A PRESENTE INVENÇÃO É UM DISPOSITIVO ELETRÓDICO PARA A DETEÇÃO DE GLICOPROTEÍNA B DO CITOMEGALOVÍRUS HUMANO, COM TRÊS ELÉTRODOS FORMADOS POR SERIGRAFIA, MODIFICANDO-SE UM DELES COM UM SISTEMA DE ANTICORPO PARA PRENDER O MESMO ANTICORPO MARCADO COM NANOPARTÍCULAS DE OURO, DISPOSTOS AMBOS EM ESTRUTURA TIPO SANDUÍCHE PARA A GLICOPROTEÍNA B, BEM COMO O PROCESSO PARA A SUA OBTENÇÃO E O SEU USO NA DETEÇÃO. A PRESENTE INVENÇÃO ENQUADRA-SE NO CAMPO DAS CÉLULAS ELETROQUÍMICAS APLICADAS À ANÁLISE DE SUBSTÂNCIAS.

RESUMO

Dispositivo eletródico para a deteção de glicoproteína B do citomegalovírus humano, processo de obtenção e uso

A presente invenção é um dispositivo eletródico para a deteção de glicoproteína B do Citomegalovírus Humano, com três elétrodos formados por serigrafia, modificando-se um deles com um sistema de anticorpo para prender o mesmo anticorpo marcado com nanopartículas de ouro, dispostos ambos em estrutura tipo sanduíche para a glicoproteína B, bem como o processo para a sua obtenção e o seu uso na deteção.

A presente invenção enquadra-se no campo das células eletroquímicas aplicadas à análise de substâncias.

DESCRIÇÃO

Dispositivo eletródico para a deteção de glicoproteína B do citomegalovírus humano, processo de obtenção e uso

Domínio técnico da invenção

A presente invenção encontra-se no campo das células eletroquímicas aplicadas à análise de substâncias.

A invenção refere-se a um dispositivo eletródico para deteção de GLICOPROTEÍNA B do citomegalovírus humano (HCMV), nomeadamente o seu processo de fabrico e respetiva utilização na deteção citada. O referido dispositivo é constituído por três elétrodos, todos os elétrodos produzidos por serigrafia, modificando-se um deles com um anticorpo.

Sumário da invenção

A presente invenção diz respeito a um dispositivo eletródico para deteção de GLICOPROTEÍNA B do citomegalovírus humano (HCMV).

Estado da técnica

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

Uma das técnicas mais utilizadas no fabrico dos elétrodos que se utilizam nas células eletroquímicas é a serigrafia.

Esta técnica permite construir sensores químicos com uma alta reprodutibilidade e uma infraestrutura mínima.

A serigrafia é um método de impressão direta, também denominado de impressão por penetração. A deposição de tintas é realizada por camadas sobre um substrato. A qualidade dos sensores químicos assim fabricados depende, em grande medida, dos materiais utilizados.

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

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

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

Atualmente, o diagnóstico de citomegalovírus humano (HCMV) é baseado em informações clínicas e imunológicas. Existem vários métodos para a deteção de HCMV. O isolamento do vírus em cultura de fibroblastos é o método convencional. Neste, é feito o isolamento do vírus a partir de um tecido de biopsia ou de um fluido corporal, tal como a urina. Após isolamento, o HCMV é replicado "*in vitro*", incubado com fibroblastos a 36 °C durante uma a três semanas e

posteriormente a incubação é analisada com o objetivo de identificar inclusões de HCMV em fibroblastos. Este método, que requer assepsia total, não é utilizado pois requer um longo período de tempo para sua execução, dificultando assim o diagnóstico.

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

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

Outra técnica é a de ELISA ("Enzyme-Linked Immunosorbent Assay") que apresenta uma sensibilidade de 100% e uma especificidade de 86% na deteção de anticorpos no sangue. No entanto, existe a possibilidade de resultados falso-

positivos, causados por reações cruzadas com algum vírus da família *Herpesviridae*, fator reumatoide e anticorpos antinucleares.

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

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

Yamamoto et al (1998) referem um diagnóstico de infeção congénita e perinatal por citomegalovírus utilizando a reação em cadeia da polimerase. A presente invenção destaca-se daquela, na medida em que na presenta invenção é usado um dispositivo electródico e nesta a deteção da glicoproteína é efetuada através de um aparelho de PCR. Nos outros documentos encontrados, Chen et al. (2009) que referem "Protein Chips and nanomaterial for application in tumor marker immunoassays"; Pingarron et al. (2008) que referem "Gold nanoparticle-based electrochemical biosensors"; Xiaodong et al. (2011) que referem "Gold nanoparticles-based signal amplification for biosensing"; Schuler et al. (2009)que referem "Screen printing as cost-efficient fabrication method for DNA-chips with electrical readout for detection of viral DNA", verifica-se que em nenhum é possível encontrar qualquer indicação de que a modificação da superfície do elétrodo de trabalho para uma estrutura tipo

sanduíche (anticorpo/glicoproteína B/anticorpo marcado com ouro) permite detetar a glicoproteína B e, assim, detetar a presença de citomegalovírus humano.

Na literatura são encontrados poucos relatos que podem ser associados à deteção de HCMV. Dois deles estão relacionados com o reconhecimento de sequências de ADN amplificadas do HCMV, através da utilização de sensores miniaturizados. Azek at al., propuseram um sensor de ADN baseado em elétrodos de carbono serigrafados. O ADN alvo é adsorvido e hibridizado com uma sonda de ADN biotinilado e a extensão de híbridos formados é depois determinada pela streptavidina conjugada com peroxidase de rábano. A intensidade da corrente do pico voltamétrico resulta da redução do produto gerado pela enzima e está relacionado com as moléculas de ADN amplificado presentes na amostra. A marcação de um oligonucleótido por uma enzima não é uma via simples, e uma vez realizada a marcação, a atividade do conjugado deve ser periodicamente controlada devido à fraca estabilidade das enzimas. Para ultrapassar essa limitação, Authier et al. exploraram uma estratégia baseada na utilização de marcadores de nanopartículas de ouro para a quantificação de uma sequência amplificada de 406 pares de base de HCMV. O ensaio baseia-se na hibridização de uma cadeia simples de ADN de HCMV com uma sonda de oligonucleótidos modificados com nanopartículas de ouro. De seguida, a dissolução oxidativa do ouro permite a determinação indireta dos iões de Au III solubilizados por voltametria de redissolução anódica. Os métodos acima descritos são usados em conjugação com a técnica de PCR para

a quantificação e ampliação dos produtos, tornando estes métodos bastante dispendiosos.

Um sensor piezoelétrico de afinidade foi descrito por Susmel et al. para a deteção da glicoproteína B do HCMV. Neste estudo, foram imobilizados anticorpos num elétrodo de ouro. Embora esta técnica não se baseia na amplificação de ADN, também requer a utilização de instrumentação dispendiosa para a sua implementação.

Mais recentemente em França, um consórcio desenvolveu um dispositivo para o rastreio do HCMV. Neste, a amostra biológica é aplicada numa superfície de ouro coberta com anticorpos específicos para o HCMV. Se o vírus estiver presente na amostra é capturado na superfície pelos anticorpos. Após a injecçao de uma sonda fluorescente, é detetado um sinal. Quanto maior a carga viral, maior a intensidade da fluorescência. Contudo, a grande desvantagem deste dispositivo é a sua baixa sensibilidade para amostras com uma baixa carga viral.

A presente invenção supera estas desvantagens através de um dispositivo eletródico, cuja simplicidade, baixo custo e tempo de análise relativamente curto, em comparação com as outras técnicas, tem levado ao incremento da sua utilização em muitos campos. Mais, esta invenção baseia-se na afinidade do sistema antigénio/anticorpo para o desenvolvimento de um imunossensor para a glicoproteína B do HCMV.

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

100% dos indivíduos infetados com HCMV desenvolvem anticorpos contra esta proteína.

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

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

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

A presente invenção combina as vantagens dos dispositivos eletródicos com o uso de anticorpos específicos para a deteção de glicoproteína B.

Descrição geral da invenção

A presente invenção é definida e caracterizada nas reivindicações independentes, enquanto as reivindicações dependentes descrevem outras características da mesma.

Com base no anteriormente enunciado, a presente invenção refere-se a um dispositivo eletródico para a deteção de glicoproteína B do HCMV. Dispositivo electródico para deteção de glicoproteína B do HCMV caracterizado por compreender três elétrodos, cada um deles composto por um contato, uma extensão e uma área ativa, sendo um o elétrodo de trabalho, outro o de referência e o último o auxiliar. Todos os elétrodos são produzidos por serigrafia de uma tinta de prata sobre uma lâmina plástica de poliéster, com exceção das suas áreas ativas. As áreas ativas dos elétrodos auxiliar e de trabalho, são impressas com uma pasta ou tinta de carbono, e a área ativa do elétrodo de referência é impressa com tinta de Ag/AgCl. Sendo, ainda, o elétrodo de trabalho modificado na sua superfície com o anticorpo anti-gB do HCMV e utilizada BSA para evitar ligações não específicas.

O dispositivo referido inclui uma região isolante que cobre as secções dos elétrodos.

Igualmente, a presente invenção refere-se a um processo de fabrico do dispositivo acima mencionado, que compreende as seguintes etapas:

- impressão por serigrafia de uma lâmina de poliéster com tinta de prata com as formas dos contactos, das extensões e dos três elétrodos,
- impressão por serigrafia da área ativa de dois dos elétrodos com tinta/pasta de carbono,
- impressão por serigrafia da área ativa de um dos elétrodos com Ag/AgCl,
- impressão por serigrafia do isolante cobrindo todas as extensões dos elétrodos,
- modificação da área ativa de um dos elétrodos mediante imobilização do anticorpo para formar uma estrutura tipo sanduíche de anticorpo/glicoproteína B do HCMV /anticorpo marcado com nanopartículas de ouro segundo o protocolo seguinte:

o eléctrodo de trabalho é inicialmente incubado com 3 µL de anticorpo anti-gB de HCMV durante 60 minutos à temperatura ambiente e mais 60 minutos a 4 °C. Após a sua lavagem com o tampão de lavagem (Tris-HNO_3 pH 7,2 e Tween 20) e Tris- HNO_3 pH 7,2, é depositada uma gota de 3 µL de solução de bloqueio (albumina sérica bovina (BSA) 5 mg/ml) e realizada novamente incubação durante 60 minutos à temperatura ambiente, seguida de lavagem com tampão de lavagem e com Tris-HNO_3 pH 7,2. Posteriormente adicionam-se 3 µL de solução de glicoproteína B de HCMV e é realizada igualmente incubação durante 60 minutos seguida de lavagem com tampão de lavagem e com Tris-HNO_3 pH 7,2. Em continuação, adicionam-se 3 µL da solução de anticorpo anti-gB de HCMV marcado com nanopartículas de ouro e procede-se à incubação durante mais 60 minutos. Após lavagem com o tampão de lavagem e com Tris-HNO_3 pH 7,2 uma gota de 2 µL de solução de deposição de prata é colocada na microcélula eletroquímica e incubada durante 4 minutos (na ausência de luz), seguida de lavagem com água. Todos estes

passos, quando nada dito em contrário, foram realizados a 4 °C. Finalmente é realizada uma voltametria de campo pulsado (DPV) entre -0,15 V e 0,25 a 50 mV s^{-1} em solução de KCl 1,0 M, para registar as correntes de redissolução.

E, a presente invenção também se refere à utilização do dispositivo acima mencionado, que compreende as seguintes etapas:
- união dos contactos dos elétrodos a um potencióstato;
- introdução do dispositivo numa célula eletroquímica que contém 5 ml de uma solução de KCl 1,0 M;
- aplicação de um varrimento de potencial entre -0,15 e 0,25 V a 50 mV s^{-1} entre o elétrodo de trabalho e o elétrodo auxiliar;
- registo das intensidades de corrente com imunossensores preparados com diferentes concentrações de glicoproteína B do HCMV.

Descrição detalhada da invenção

De forma detalhada, a invenção refere-se a um dispositivo eletródico para deteção de glicoproteína B do HCMV, com três elétrodos (1, 2, 3), todos os elétrodos produzidos por serigrafia, incubando um deles seguindo uma estrutura tipo sanduíche: solução de anticorpo, solução de glicoproteína B do HCMV, solução de anticorpo marcado com nanopartículas de ouro e por último solução de deposição de prata; e ao seu processo de fabricação e seu uso na deteção citada.

A invenção refere-se ao dispositivo, procedimento de fabrico e de uso de um dispositivo com três elétrodos (1, 2, 3), descartáveis, serigrafados sobre um suporte de poliéster, em concreto politerefetalato de etileno (PET), cujo arranjo

espacial permite uma análise rápida "in situ", de pequenos volumes de amostra por técnicas eletroquímicas.

O dispositivo consiste em três elétrodos serigrafados (1, 2, 3): elétrodo auxiliar (1) ou contra-elétrodo, elétrodo de trabalho (2) e elétrodos de referência (3).

Os elétrodos têm três zonas ou partes diferentes: os contactos (1.3, 2.3, 3.3) ou bornes, para sua ligação a um potencióstato comercial; as áreas ativas (1.1, 2.1, 3.1), em contato direto com a amostra a analisar, as extensões (1.2, 2.2, 3.2), como conexão entre os contactos (1.3, 2.3, 3.3), e as áreas ativas (1.1, 2.1, 3.1).

O procedimento de fabrico do dispositivo por serigrafia é levado a cabo com a ajuda de uma série de telas, moldes ou padrões de serigrafia nos quais aparece, numa superfície porosa o esquema do motivo a imprimir que será a forma dos elétrodos:

- O primeiro padrão é usado para produzir, através da serigrafia com pasta ou tinta de prata, o contra-elétrodo (1), o elétrodo de trabalho (2), o elétrodo de referência (3), os contatos (1.3, 2.3 e 3.3) e as extensões (1.2, 2.2 e 3.2). Isto é, cria-se a base condutora elétrica do dispositivo. Após a aplicação desta camada, ela deve ser curada durante 30 minutos a 120 °C.

- O segundo padrão está desenhado para a obtenção das áreas ativas (1.1 e 2.1) dos elétrodos auxiliar (1) e de trabalho (2). Para a sua formação, imprime-se uma pasta ou tinta de carbono sobre a base condutora na zona da área ativa dos

ditos elétrodos, que posteriormente se submete a um processo de cura nas mesmas condições, 30 minutos a 120 °C.

- O terceiro padrão está desenhado para a obtenção da área ativa (3.1) do elétrodo de referência (3). Para a sua formação, imprime-se um produto comercial à base de Ag/AgCl sobre a base condutora na zona da área ativa do dito elétrodo, que posteriormente se submete a um processo de cura nas mesmas condições, 30 minutos a 120 °C.

- Por último, o quarto padrão é uma camada final de tinta de isolamento que cobre as extensões (1.2, 2.2, 3.2), ficando os contactos (1.3, 2.3, 3.3) e as zonas ativas (1.1, 2.1, 3.1) dos elétrodos livres. A correta formação desta camada isolante (4) exige a cura a 80 °C durante 30 minutos.

Os três elétrodos (1, 2, 3), nesta forma de produção, são de geometria retangular. As melhores dimensões da superfície ativa (3.1) do elétrodo de referência do dispositivo correspondem a 2 mm de largura por 6 mm de comprimento.

Para o elétrodo de trabalho (2), a melhor dimensão comprovada da sua área ativa (2.1) corresponde a um quadrado de 4 mm^2.

Numa primeira produção, o elétrodo auxiliar (1) tem uma forma retangular, como se vê na Figura 1. Numa segunda produção, o elétrodo auxiliar tem uma forma arqueada, conforme mostrado na Figura 2.

O processo de alteração do elétrodo de trabalho é constituído por várias etapas de incubação:

Na primeira etapa, 3 µL de soluções de anti-gB de HCMV, são incubados no elétrodo durante 60 minutos à temperatura ambiente e mais 60 minutos a 4°C, seguindo-se lavagem com tampão de lavagem (Tris-HNO_3 pH 7,2 e Tween 20) e Tris-HNO_3 a pH 7,2;

Na segunda etapa, é depositada uma gota de 3 µL de solução de bloqueio (BSA 5 mg/mL) e realizada incubação durante 60 minutos à temperatura ambiente seguida de lavagem com tampão de lavagem e Tris-HNO_3 pH 7,2;

Na terceira etapa, é depositada uma gota de 3 µL de gB do HCMV e realizada incubação durante 60 minutos a 4 °C seguida de lavagem com tampão de lavagem e Tris-HNO_3 pH 7,2;

Na quarta etapa, são adicionados 3 µL da solução de anticorpo anti-gB de HCMV marcado com nanopartículas de ouro e procede-se a incubação durante 60 minutos a 4°C. Depois lava-se com tampão de lavagem e Tris-HNO_3 pH 7,2;

Na quinta e última etapa, 2 µL de solução de deposição de prata são adicionadas à microcélula eletroquímica, incubando durante 4 minutos a 4 °C na ausência de luz, seguido por lavagem com água.

O dispositivo assim formado é um sensor miniaturizado cuja geometria é ótima para a análise de amostras reais líquidas e de pequeno tamanho. A adição de um pequeno volume com uma micropipeta diretamente sobre o sistema eletródico permite a análise sem a necessidade de utilização de uma célula eletroquímica convencional, tornando mais fácil, rápida e económica a análise "in situ" por ser suscetível de ser

conectado a um potencióstato comercial portátil. Alternativamente, o sistema pode ser introduzido numa célula eletroquímica convencional.

Para colocar em funcionamento o dispositivo, ligam-se as partes superiores condutoras (1.3, 2.3, 3.3) de cada um dos elétrodos (1, 2, 3), com os bornes de saída de um potencióstato por conexões elétricas. Através destas e de acordo com a técnica selecionada para levar a cabo a determinação voltamétrica, executa-se um varrimento de potencial de -0,15 a 0,25 V a 50 mV s-1 e registam-se os voltamogramas obtidos. A medição é realizada à temperatura ambiente.

Para levar a cabo a determinação de glicoproteína B do HCMV numa amostra desconhecida, usa-se uma célula eletroquímica contendo 5 ml de uma solução de KCl 1 M, regista-se o voltamograma do qual se retira a intensidade de corrente e aplica-se na reta de calibração para obtenção da concentração.
A reta de calibração é previamente estabelecida com sistemas eletródicos construídos com concentrações conhecidas de glicoproteína B do HCMV.

Descrição das Figuras

Completa-se a presente memória descritiva com um conjunto de figuras, ilustrativas do exemplo preferido.

Figura 1: Representação esquemática de uma vista em alçado dos elétrodos de uma primeira realização na qual o elétrodo auxiliar tem forma retangular. Sendo 1.3, 2.3 e 3.3 os contatos; 1.2, 2.2 e 3.2 as extensões; 1.1, 2.1 e 3.1 as

áreas ativas respetivamente dos elétrodos 1, 2 e 3; e 4 o isolador.

Figura 2: Representação esquemática de uma vista em alçado dos elétrodos de uma segunda realização na qual o elétrodo auxiliar tem forma de arco. Sendo 1.3, 2.3 e 3.3 os contatos; 1.2, 2.2 e 3.2 as extensões; 1.1, 2.1 e 3.1 as áreas ativas respetivamente dos elétrodos 1, 2 e 3; e 4 o isolador.

Covilhã e UBI, 24 de junho de 2013

REIVINDICAÇÕES

1. Dispositivo eletródico para a deteção de glicoproteína B do citomegalovírus humano caracterizado por compreender três elétrodos (1, 2, 3), no qual cada elétrodo consta de um contacto (1.3, 2.3, 3.3), uma extensão (1.2, 2.2, 3.2) e uma área ativa (1.1, 2.1, 3.1), sendo um o elétrodo de trabalho (2), outro o auxiliar (1) e o outro o de referência (3), todos os elétrodos produzidos por serigrafia de uma tinta de prata sobre uma lâmina plástica de poliéster, com exceção das suas áreas ativas, sendo que as áreas ativas dos elétrodos auxiliar (1.1) e de trabalho (2.1), são impressas com uma pasta ou tinta de carbono, e a área ativa do elétrodo de referência (3.1) é impressa com tinta de Ag/AgCl, bem como, ainda, o elétrodo de trabalho (2) é modificado na sua superfície com o anticorpo anti-gB do HCMV e utilizada BSA para evitar ligações não específicas.

2. Dispositivo eletródico para a deteção de glicoproteína B do citomegalovírus humano, de acordo com a reivindicação 1, caracterizado por incluir um isolador (4) que cobre as extensões (1.2, 2.2, 3.2) de todos os elétrodos.

3. Dispositivo eletródico para a deteção de glicoproteína B do citomegalovírus humano, de acordo com a reivindicação 1, caracterizado por os contactos (1.3, 2.3, 3.3), as extensões (1.2, 2.2, 3.2) e as áreas ativas dos elétrodos de trabalho (2.1) e de referência (3.1) serem de forma retangular.

4. Dispositivo eletródico para a deteção de glicoproteína B do citomegalovírus humano, de acordo com a reivindicação 1

e 3, caracterizado por a área ativa (3.1) do elétrodo de referência ter 2 mm de largura por 6 mm de comprimento.

5. Dispositivo eletródico para a deteção de glicoproteína B do citomegalovírus humano, de acordo com a reivindicação 1 e 3, caracterizado por a área ativa (2.1) do elétrodo de trabalho (2) ser um quadrado de 4 mm^2.

6. Dispositivo eletródico para a deteção de glicoproteína B do citomegalovírus humano, de acordo com a reivindicação 1, caracterizado por a área ativa (1.1) do elétrodo auxiliar ser retangular.

7. Dispositivo eletródico para a deteção de glicoproteína B do citomegalovírus humano, de acordo com as reivindicações 1 e 6, caracterizado por a área ativa (1.1) do elétrodo auxiliar poder ser em forma de arco.

8. Processo de obtenção do dispositivo eletródico para a deteção de glicoproteína B do citomegalovírus humano, de acordo com as reivindicações 1 a 7, caracterizado por compreender os seguintes passos:

a) impressão por serigrafia de uma lâmina de poliéster com tinta de prata com as formas dos contactos (1.3, 2.3, 3.3), das extensões (1.2, 2.2, 3.2) e dos três elétrodos (1, 2, 3);
b) impressão por serigrafia da área ativa de dois dos elétrodos (1.1 e 2.1) com tinta/pasta de carbono;
c) impressão por serigrafia da área ativa de um dos elétrodos (3.1) com Ag/AgCl;

d) impressão por serigrafia do isolante (4) cobrindo todas as extensões (1.2, 2.2, 3.2) dos elétrodos;
e) modificação da área ativa de um dos elétrodos (2.1) mediante adsorção sequencial de anticorpos anti-gB do HCMV e de BSA na sua superfície.

9. Uso do dispositivo eletródico para a deteção de glicoproteína B do citomegalovírus humano, de acordo com a reivindicações de 1 a 8, caracterizado por compreender os seguintes passos:

a) incubação de 3 μL de solução de gB do HCMV na superfície do elétrodo de trabalho;
b) incubação 3 μL de solução do anticorpo anti-gB do HCMV marcado com nanopartículas de ouro na superfície do elétrodo de trabalho;
c) aplicação de 2 μL de solução de deposição de prata na superfície do elétrodo de trabalho;
d) união dos contactos (1.3, 2.3, 3.3) dos elétrodos a um potencióstato;
e) introdução do dispositivo numa célula eletroquímica que contém 5 mL de uma solução KCl 1M;
f) aplicação de um varrimento de potencial entre -0.15 e 0.25 V entre o elétrodo de trabalho (2) e o elétrodo auxiliar (1);
g) Registo das intensidades de corrente.

Covilhã, 24 de junho de 2013

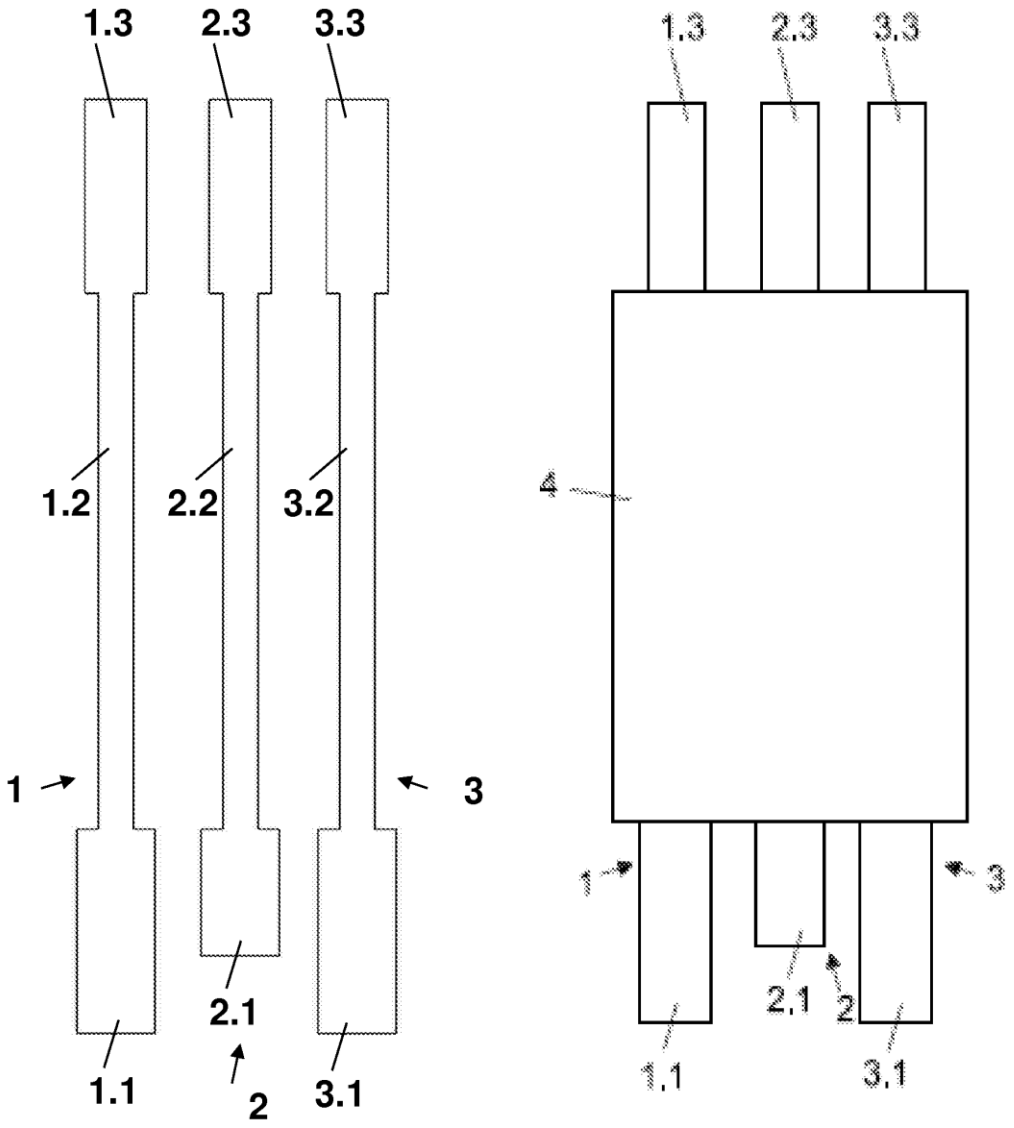


Fig.1

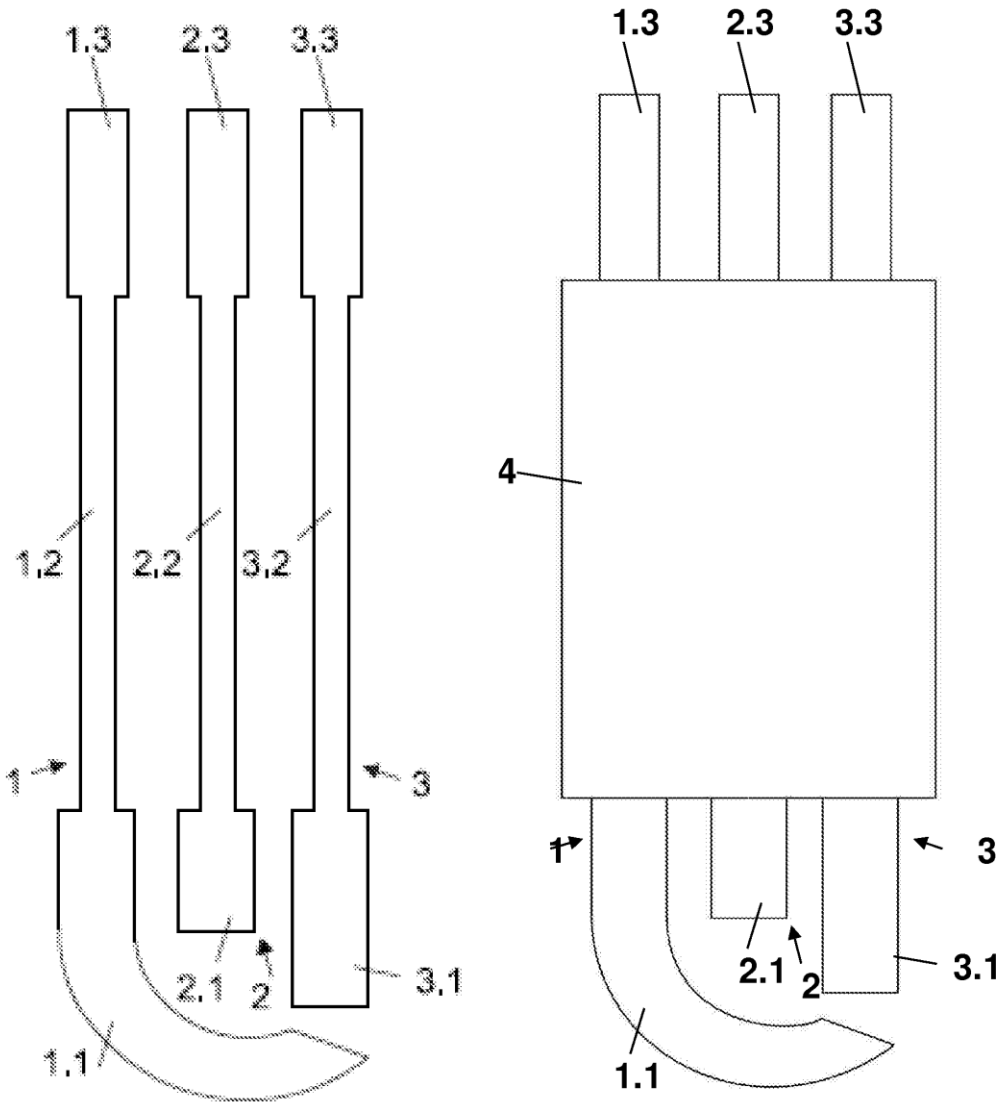


Fig.2