UNIVERSIDAD POLITÉCNICA DE MADRID

ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRONÓMICA,
ALIMENTARIA Y DE BIOSISTEMAS

**“Enhancing the biological activity of peptides by
chemical or biological functionalization of *Turnip mosaic
virus*-derived nanoparticles.”**

TESIS DOCTORAL

Ivonne González Gamboa
Ingeniera en Biotecnología

2017

Universidad Politécnica de Madrid

02. Escuela Técnica Superior de Ingeniería Agronómica,
Alimentaria y de Biosistemas

Tesis Doctoral:

**“Enhancing the biological activity of peptides by chemical or
biological functionalization of *Turnip mosaic virus*-derived
nanoparticles.”**

Autor:

Ivonne González Gamboa

Ingeniera en Biotecnología

Director:

Fernando Ponz Ascaso

Doctor en Bioquímica y Biología Molecular

Madrid, 2017

Tribunal nombrado por el Magfco. Y Excmo. Sr. Rector de la Universidad Politécnica de Madrid el día

Presidente: Fernando García-Arenal Rodríguez

Secretaria: Araceli Díaz Perales

Vocal: María del Sol Cuenca Martín

Vocal: José Antonio Darós Arnau

Vocal: Ana María Pizarro Arranz

Suplente 1: Marina Inmaculada Garín Ferreira

Suplente 2: Laura Benítez Rico

ACKNOWLEDGMENTS

ABBREVIATIONS

3D – three dimensional

AP – Alkaline phosphatase

BSA – bovine serum albumin

CMV – Cowpea mosaic virus

CP – coat protein

dpa – days post-agroinfiltration

dpi – days post-inoculation

DTT - Dithiotreitol

ELISA – Enzyme-Linked ImmunoSorbent Assay

GFP – Green fluorescent protein

HRP – Horseradish peroxidase

I-AEDANS - 5-[2-(Iodoacetamido)ethylamino]naphthalene-1-sulfonic acid

Ig - Immunoglobulin

ISEM – Immunosorbent electron microscopy

kDa – kilodaltons

LB – Luria Betani

mRNA – messenger ribonucleic acid

MAB – monoclonal antibody

nt - nucleotide

OD₆₀₀ – Optical density at 600

PBS – Phosphate buffered saline

PDB – Protein data bank

PEG – Polyethylene glycol

PLA – polylactic acid

pI – isoelectric point

PMMV – Pepper mottle mild virus

PVA – Potato virus A

PVY – Potato virus Y

rpm – revolutions per minute

RT – room temperature

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

SMV – Soybean mosaic virus

ssRNA – single-stranded ribonucleic acid

Sulfo-SIAB - sulfosuccinimidyl[4-iodoacetyl]aminobenzoate

TEM – transmission electron microscopy

TMV – Tobacco mosaic virus

TRAP – Thrombin receptor activator peptide

TuMV – Turnip mosaic virus

VIP – vasoactive intestinal peptide

VLP – Virus-like particle

VNP – Viral nanoparticle

VEGFR-3 – Vascular endothelial growth factor 3

WB – Western Blot

INDEX

RESUMEN

ABSTRACT

1. INTRODUCTION

1.1 Nanobiotechnology

1.2 An overview on plant viruses

1.2.1 Potyvirus

1.2.2 Virus-like particles

1.2.2.1 VLP production systems

1.3 VNPs as displaying systems

1.3.1 Genetic functionalization

1.3.1.1 Limitations

1.3.2 Chemical functionalization

1.3.2.1 Limitations

2. OBJECTIVES

3. RESULTS

3.1 Plant-made potyvirus-like particles used for log-increasing antibody sensing capacity

3.2 Detection of autoantibodies to Vascular Endothelial Growth Factor Receptor-3 in Bile Duct Ligated rats and correlations with a panel of traditional markers of liver diseases

3.3 Chemical conjugation of peptides and low-molecular-weight molecules on the surface of *Turnip mosaic virus* nanoparticles

4. GENERAL DISCUSSION

5. CONCLUSIONS

6. REFERENCES

7. ANNEX (PUBLISHED PAPERS)

RESUMEN

En esta tesis se desarrollaron nanopartículas hechas en planta, derivadas del *Virus del mosaico del nabo* (TuMV, por sus siglas en inglés), un potivirus. Estas nanopartículas virales (VNPs) pueden ser explotadas como armazones para construir nano-objetos modificados que pueden ser usados como herramientas de diagnóstico; para la generación de anticuerpos; inmunización; o como biomateriales. Estas entran en dos categorías biológicas: las que contienen ácidos nucleicos (viriones) y las que no (partículas “tipo” virus vacías, eVLPs). Las modificaciones que se pueden realizar en las VNPs son a través de dos aproximaciones: conjugación química o genética. Cada una cuenta con sus pros y sus contras para su uso nanotecnológico. En general, este es el primer caso de modificación genética o química en VNPs de potyvirus hechas en planta. Las VNPs fueron producidas por inoculación de mostaza india (*Brassica juncea*) con el virus de TuMVm, o por la agroinfiltración de *Nicotiana benthamiana* con el cDNA de la proteína de la cápside (CP) de TuMV en un vector pEAQ para la alta expresión de proteína. Como prueba de concepto para la modificación genética se seleccionó un péptido del receptor de la trombina (TR) y para la química, un péptido vasoactivo intestinal y compuestos de bajo peso molecular (biotina, organometálicos). Producción significativa de TR-CP eVLPs y viriones de TuMV fueron obtenidos de hojas de planta. Las VNPs fueron observadas en un microscopio electrónico tras inmunodecorarlos con anticuerpo anti-TR o anti-biotina, respectivamente. Ambas VNPs fueron capaces de incrementar por órdenes de magnitud la habilidad de sensar anticuerpos, comparadas con péptido o biotina libre.

En esta tesis se demuestra la posibilidad de conjugar, genética- o químicamente, péptidos y compuestos de bajo peso molecular a viriones del *Virus del mosaico* del nabo, o sus eVLPs. Esto muestra el potencial de estas VNPs por su uso en numerosas aplicaciones nanobiotecnológicas.

ABSTRACT

In this thesis, plant-made nanoparticles derived from *Turnip mosaic virus* (TuMV), a potyvirus, were developed. These Viral Nanoparticles (VNPs) can be exploited as scaffolds for building modified nano-objects and can be used as diagnostic tools, for antibody generation, immunization, or as biomaterials in general. They fall into two biological categories: either enclosing a nucleic acid (virions) or without the nucleic acid (empty virus-like particles, eVLPs). The modifications imposed on VNPs can also be achieved by two main approaches: genetic or chemical conjugation. Each of them has particular pros and cons for nanotechnological deployment. Overall, this is the first case of genetic or chemical conjugation in plant-made potyvirus VNPs. The VNPs were produced either by inoculation of Indian mustard (*Brassica juncea*) with the infectious TuMV virus, or by agroinfiltration of the tobacco relative *Nicotiana benthamiana* with the TuMV coat protein cDNA (CP) on a pEAQ vector for high protein expression. As a proof-of-concept, a human thrombin receptor peptide (TR) for the genetic modification, and a vasoactive intestinal peptide and low-molecular-weight compounds (biotin, organometallics) for the chemical modification, were selected. Significant production of TR-CP VLPs and TuMV virions was obtained from plant leaves. VNPs were observed under an electron microscope after immunodecoration with anti-TR antibody or anti-biotin antibody, respectively. Both VNPs proved capacity to log-increase the ability of antibody-sensing compared to free TR peptide or free biotin.

In this thesis, the possibility of conjugating, genetically and chemically, peptides and low-molecular-weight compounds to *Turnip mosaic virus* virions or VLPs is demonstrated. This outlines the potential of these VNPs for their use in numerous nanobiotechnological applications.

1. INTRODUCTION

1.1 NANOBIO TECHNOLOGY

Nanotechnology is an emerging field that covers a wide range of disciplines, including the frontiers of chemistry, materials, medicine, electronics, optics, sensors, information storage, communication, energy conversion, environmental protection, aerospace and more. It focuses on the design, synthesis, characterization and application of materials at the nanoscale (Meier 1999, Yin, Shah et al. 2015). Nanomaterials are the foundation of nanotechnology and are anticipated to open new avenues to numerous emerging technological applications. This field has grown very fast in the past two decades because of the availability of new approaches and tools for the synthesis, characterization, and manipulation of nanomaterials. The research on nanomaterials is highly interdisciplinary because it involves many different synthetic methodologies and characterization techniques.

Integration of nanotechnology with biotechnology is expected to accelerate in the next decade (Roco 2003). In particular, nanobiotechnology integrates nanoscale principles and techniques with biological systems in the development of nanostructures, design tools and devices to facilitate biomedical research, and generate new therapeutics (Kim, Lee et al. 2008), among other applications.

Reasons for the widespread use of nanosized objects are multifold. Despite their smallness, nanosized objects (1-1000 nm in diameter) are still considerably larger than molecules, and hence can receive surface engraftment of different types of moieties to achieve multiple functions simultaneously. The smallness also translates to a high surface area to volume ratio, allowing more dense and compact packing of moieties on their periphery.

The increased interest about the nanomaterials applications in a huge range of disciplines has generated fast growing concerns about the potential toxicological, health and ecological effects of the nanomaterials. In fact the health and environmental issues related to the design and applications of the nanomaterials are highly complicated. The toxicological, health and

environmental effects do not depend only on the nature or type of material, but also on the size, shape, quality and colloidal stability, surface modification and surface charges, the medium (solvent, buffer or biological media) and a wide range of other physicochemical properties as well as the continuous changes and transformation of the nanomaterials due to the interaction with the surrounding (Zhang, Xu et al. 2012).

A variety of materials exist from which nanoparticles can be synthesised. Some inorganic materials such as silica and iron oxide nanoparticles have shown potential as a delivery system (Tang, Li et al. 2012, Mody, Cox et al. 2014), however their toxicity and clearance from the body raises a few concerns (Vallhov, Gabrielsson et al. 2007, Wang, Sanderson et al. 2007, Liu, Li et al. 2011, Kang and Lim 2012). Therefore, more biocompatible and biodegradable materials have gained interest. Numerous nanoparticle systems are available, like biodegradable polymeric poly (lactic-co-glycolic acid) and polylactic acid (PLA) nanoparticles, liposomes, natural polymers like chitosan and biological nanoparticles such as viral nanoparticles (VNPs) (Hansen and Lehr 2012). The latter (VNPs) are natural nanomaterials and have recently received attention for their tremendous potential.

Viruses serve as excellent nano-building blocks for materials design and fabrication. The main advantages besides their nanometer-range size are the propensity to self-assemble into monodisperse nanoparticles of discrete shape and size, the high degree of symmetry and polyvalence, the relative ease of producing large quantities, the exceptional stability and robustness, biocompatibility, and bioavailability.

1.2 AN OVERVIEW ON PLANT VIRUSES

Viruses are sub-microscopic, obligate intracellular parasites that are not functionally active outside their host cells. Like all biological entities, viruses possess genes, reproduce themselves and have the ability to adapt to changing environments. A virus is a form of life with very simple

requirements. Its basic needs are a nucleic acid to be transmitted from generation to generation and a messenger RNA to direct the synthesis of viral proteins.

To date, the total number of viruses is known to exceed 4000, and new viruses are continuously described. About one fourth of all known viruses have been described from plants (Agrios 2005). They, also, have a great impact on us humans. Indeed, plant viruses cause important diseases in crop plants resulting in enormous economic losses. Also, recently these plant viruses have been used as scaffolds (in icosahedral, rod-shaped or flexuous elongated form) for applications in the biomedical or pharmaceutical industries, among others (Fig. 1) (Smith, Lindbo et al. 2006, Dedeo, Finley et al. 2011, Geiger, Eber et al. 2013, Koch, Wabbel et al. 2015).

Fig. 1. Structures of a selection of plant viruses that have been used as virus-based nanoparticles. Structural data for icosahedral viruses were obtained from <http://viperdb.scripps.edu/>, and structural

data for TMV were obtained from <http://www.rcsb.org>; images were produced using Chimera software. For PVX and TuMV, schematic representations of the virus particles are shown. Modified from Koudelka et al, 2015.

1.2.1 TURNIP MOSAIC VIRUS

The family Potyviridae, along with the Picornaviridae, Sequiviridae, Comoviridae and Caliciviridae families, form the “picorna-like group” of positive-sense, single-stranded RNA viruses defined on the similarities in the genome organization and expression strategy (Goldbach, 1986). Within this group, the family Potyviridae contains six established genera: Potyvirus, Macluravirus, Bymovirus, Rymovirus, Tritimovirus, and Ipomovirus (Fauquet and Fargette 2005). Potyviruses are transmitted by aphids in a non-persistent manner (Shukla, Ward et al. 1994) and are the largest and economically most important group of plant infecting viruses (Fauquet and Martelli 2001). Currently, the genus Potyvirus contains 118 definite and 82 tentative species (Fauquet and Martelli 2001), which represent more than 30% of all known plant viruses.

The potyviral genome consists of a single-stranded (ss) positive-sense RNA molecule of about 10 kilobases. Its virions are flexuous rods built up from approximately 2000 identical coat protein (CPs) and a single RNA molecule (Walsh and Jenner, 2002). The genome contains a single open reading frame (ORF), flanked by a NTR at both (5' and 3') ends. The ORF encodes a large polyprotein that is co- and/or post-translationally cleaved by three viral-encoded proteinases (P1, HC-Pro and NIa-Pro) (Riechmann, Lain et al. 1992). The P1 and HC-Pro mediate their own cleavage from the polyprotein (Carrington, Freed et al. 1989, Verchot, Koonin et al. 1991) whereas the NIa-Pro is responsible for the cleavage of the C-terminal two-thirds of the polyprotein.

Turnip mosaic virus belongs to this Potyvirus genus. Its virions are flexuous filaments without an envelope and have a diameter of 12 to 15 nm and a length of 700 to 800 nm

approximately. The coat protein (33 kDa in size) is a three domain protein with N- and C-terminus regions exposed on the virion's surface (Urcuqui-Inchima, Haenni et al. 2001).

1.2.2 VIRUS-LIKE PARTICLES

Virus-like particles (VLPs) are composed of viral coat proteins that when expressed using recombinant expression systems, the viral structural proteins intrinsically self-assemble into hollow particles resembling the parental virus (Burrell, Mackay et al. 1979, Delchambre, Gheysen et al. 1989). However, they do not contain viral nucleic acid and are, consequently, unable to replicate and cause a disease (Grgacic and Anderson 2006, Roy and Noad 2009).

Nowadays, more than 100 VLPs from 35 different virus families originating from microbial, plant, insect and mammalian viruses (Zeltins 2013) have been characterized, clearly highlighting their versatility and increasing scientific and therapeutic interest.

1.2.3 VLP PRODUCTION SYSTEMS

There are many expression systems for the production of VLPs, such as various species of yeast that include *Saccharomyces cerevisiae* and *Pichia pastoris*; bacteria, mainly *Escherichia coli* (*E. coli*), the baculovirus expression vector/insect cell system (BEVS/IC), various mammalian cell lines (Grgacic and Anderson 2006) as well as plants, like tobacco or lettuce leaves (Mason, Ball et al. 1996, Kong, Richter et al. 2001).

Plant expression systems have many advantages compared to mammalian cells. Not only are plant production platforms cost-effective, highly scalable, and free of mammalian pathogens, but protein folding, assembly and post-translational modifications in plants also resemble those produced in mammalian cells (Kushnir, Streatfield et al. 2012). Also, there are vectors like the pEAQ vectors that allow high-level expression of proteins within a few

days(Sainsbury, Thuenemann et al. 2009), making the production of VLPs through this methodology a time efficient approach (Fig. 2).

Fig. 2. Schematic representation of the production of VLPs.

1.3 VIRAL NANOPARTICLES (VNPs) AS DISPLAY SYSTEMS

The CP of TuMV can be used as scaffold for the display of peptides or low-molecular-weight compounds. The display can be achieved through the incorporation of molecules onto or into the VNPs either by genetic fusion into subunit proteins of the capsid (chimeric VNPs) or by chemical conjugation to preformed VNPs (conjugated or coupled VNPs). In such cases, the VNPs serve both as a presentation scaffold for extraneous antigens (Bárcena and Blanco 2013). Other applications of chimeric VNPs are as carriers of various encapsidated therapeutic or diagnostic agents, such as proteins or nucleic acids, as adjuvants for vaccines or gene therapy, as carriers

of small drug molecules to be delivered to specific cells, and in targeting to desired organs, tissues, or cells (Pushko, Pumpens et al. 2013).

The use of VNPs as carriers of foreign molecules is not limited to those VNPs that originate from economically significant viruses or viruses that relate to the condition to be treated. The VLPs derived from hepatitis B core and surface antigens (HBcAg, HBsAg) and bacterial phage Q β are the most extensively investigated chimeric carriers of foreign antigens to date (Pushko, Pumpens et al. 2013). Almost all efforts regarding the production in planta of VNPs displaying fused epitopes on their surfaces are from icosahedral viruses (Santi, Huang et al. 2006, D'Aoust, Lavoie et al. 2008, Santi, Batchelor et al. 2008). Reports from elongated viruses are mainly from rod-shaped viruses like *Tobacco mosaic virus* (TMV). However, very little is known from flexuous elongated VNPs like TuMV (Gonzalez 2011, Ibort 2011, Sanchez, Saez et al. 2013), although they offer advantages like bigger area for peptide display, hence allowing more peptides fused to the CP than in icosahedral viruses.

1.3.1 GENETIC FUNCTIONALIZATION

The most common method for displaying foreign epitopes on VNPs is genetic insertion of target sequences into capsid proteins to form chimeric VNPs. Chimeras are produced by cloning the DNA of epitopes into the genes that encode the CP. Upon assembly of the chimeric subunit proteins, the introduced epitopes are presented on the VNP surface in a repetitive and ordered fashion, at relatively high density (one copy per subunit), and ideally in the right conformation (Jennings and Bachmann 2008).

1.3.1.1 LIMITATIONS FOR GENETIC FUNCTIONALIZATION

The key to this technology is the knowledge of the detailed structure of the VNP in order to identify the suitable insertion sites within the primary sequence of the VNP subunits to incorporate the target epitopes. The correct folding and assembly and, therefore, the structural integrity and immunogenicity of the VNP should not be compromised by the insertion. Often the easiest and least invasive site for the insertion is at the N- or C-terminus of the VNP protein subunit, especially if the three-dimensional (3D) structure of the VNP is not available.

Still, it is difficult to predict whether the introduced peptides will be compatible with VNP assembly at the intended insertion site. In addition to peptide insertions with high hydrophobicity, a strong positive charge or high β -strand index that are likely to be problematic for VNP assembly (Bárcena and Blanco 2013). Also, the size of the peptide insert is usually restricted to less than 20-30 amino acids.

1.3.2 CHEMICAL CONJUGATION

Genetic fusion has been successful in numerous examples but remains a time-consuming trial-and-error approach (Mateu 2011). As stated above, the use of chimeric VNPs has been limited, because the self-assembly of chimeras is often unpredictable, the length of the fused antigen is restricted, non-peptide antigens cannot be inserted and the folding of the introduced antigen is often compromised, which means that conformational epitopes may not be preserved. Therefore, separately expressing the VLP and target antigen is often preferable or necessary; subsequently the VNP and target antigen must be linked together. The advantages of this approach are that the size and structure of the antigen to be attached is not limited by the requirements of the correct folding and assembly of the VNP subunits, and that diverse kinds of molecules can be attached. Examples of such molecules are short peptides (Sanchez, Saez et al. 2013), full-length proteins (Bárcena and Blanco 2013), or even non-peptide antigens,

such as glycopolysaccharides (Kaltgrad, O'Reilly et al. 2008), and small organic molecules (Cornuz, Zwahlen et al. 2008).

Chemical coupling can be addressed basically to any reactive amino acid present on the surface of the VNP. The most common bioconjugation techniques (Fig. 3) are: acylation of the amino groups of lysine side chains and the N -terminus, alkylation of the sulfhydryl group of cysteine, and, to a more limited extent, activation of carboxylic acid residues and coupling with added amines (Hermanson 2013). Furthermore, VLPs can be engineered by targeted mutagenesis to contain suitable reactive amino acids on the surface of the particle (Strable and Finn 2009).

Fig. 3. Traditional bioconjugation methods used for covalent modification of virus particles. Acylation of amino groups, usually with N-hydroxysuccinimide esters or isothiocyanates (blue); alkylation of thiol groups, usually with maleimides or bromo/iodo acetamides (red); activation and capture of carboxylic acid groups using carbodiimides (usually 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, EDC) and amines (green). (Modified from Strable and Finn, 2009).

1.3.2.1 LIMITATIONS FOR CHEMICAL CONJUGATION

Like genetic modifications, chemical conjugation also has limitations regarding the size of the peptide/protein/compound attached to the VNPs. Another concern is the linking site and its spatial configuration in the coat protein. Steric hindrances are often an issue. Since no crystallization of potyviruses has been done, it is difficult to address this beforehand. Nonetheless, recent viral models and cryoEM images have elucidated this issue.

2. OBJECTIVES

The main aim of this study was to functionalize virions and virus-like particles derived from *Turnip mosaic virus* for different applications. More specifically, the aims of the study were:

1. To produce virions and virus-like particles from *Turnip mosaic virus* in *Brassica juncea* and *Nicotiana benthamiana*, respectively; and to develop suitable purification method for the VLPs.
2. To characterize the produced VLPs and to study whether a modified RNA can be encapsulated into the VLP.
3. To produce chimeric VLPs by introducing a genetic modification to the TuMV VLP structure.
4. To introduce the first *Turnip mosaic virus* CP model.
5. To chemically conjugate low-molecular-weight compounds and peptides to the surface of the TuMV virions and VLPs.
6. To evaluate the antibody sensing capacity of the chimeric VNPs (either genetically or chemically modified) for different applications.

3. RESULTS

Part 1. Genetic functionalization

Plant-made potyvirus-like particles used for log-increasing antibody sensing capacity

ABSTRACT

Deployment of the elongated flexuous virions of *Turnip mosaic virus* (TuMV), a potyvirus, for peptide display on their external surface has been previously reported by us. Nonetheless, both in TuMV and other potyviruses some peptides hinder the ability of the virus to infect host plants. We found that a peptide derived from the human thrombin receptor (TR) inhibited TuMV infectivity. In an effort to get around this problem, TuMV virus-like particles (VLPs) were produced in plants by transient high-level expression of wild-type or recombinant coat protein (CP). Significant production of both recombinant and non-recombinant CP proteins was obtained from plant leaves. Assembled particles of each of these two proteins into VLPs were observed under the electron microscope. The capacity of TR-CP VLPs to log-increase the ability of TR antibody-sensing was confirmed. These results confirm that the use of VLPs is an effective way to overcome the problem of displaying infectivity-interfering peptides. This is yet another way of exploiting the use of plant-made flexuous elongated VLPs for nanobiotechnological purposes.

INTRODUCTION

Viral nanobiotechnology is a multidisciplinary field comprising virology, biotechnology, material science, chemistry and biomedicine, and has encouraged the exchange of concepts

and methods between these disciplines. It proposes the use of viruses, as custom fitted nanoscale building materials.

Viral nanoparticles (VNPs), a term enclosing virions and virus-like particles (VLPs), have been used as scaffolds that expose peptides on their surface (Johnson, Lin et al. 1997, Fernández-Fernández, Martínez-Torrecuadrada et al. 1998, Bendahmane, Koo et al. 1999, Jiang, Li et al. 2006, Kumar, Ochoa et al. 2009, Tissot, Renhofa et al. 2010, Sanchez, Saez et al. 2013), which could be genetically fused or chemically conjugated. The system is attractive because viruses contain a large number of coat protein (CP) subunits. As new biomaterials, VNPs are interesting because they are nanoscale particles, generally symmetrical and monodisperse and can also be readily produced in large quantities in the laboratory, or even at industrial or semi-industrial levels. Like the areas that the field encompasses, the applications of this emerging technology are equally of a broad spectrum. They expand from the more obvious examples like biomedicine and agriculture, to the more engineering-oriented approaches like micro and nanodevices, including electronic applications, and certainly these will increase as the field develops.

There are several systems in which to produce these VNPs such as bacteria, insects, yeast, avian, and mammalian cells or plants (Kushnir, Streatfield et al. 2012). Nonetheless, the advantages of *in planta* expression include the pathogenic innocuousness of plant viruses to humans or animals, the low cost and high speed of generating large amounts of tissue, as well as the relatively ease of purification from infected plants (Giddings 2001, Grill, Palmer et al. 2005). The molecular farming concept of using plants as natural biofactories of biomolecules is thus enlarged with the production of tailor-made nanoparticles, the modified VNPs.

Until recently, almost all data referred to the production of icosahedral VNPs (Huang, Elkin et al. 2005, D'Aoust, Lavoie et al. 2008, Santi, Batchelor et al. 2008). However, working with icosahedral viruses limits the space available for peptide display due to the geometry of

the particles. In order to display more units per particle, elongated viruses can be used. Their advantage is that their capsids contain a large number of coat protein copies with helical symmetry. However, the ability to express a peptide on a viral surface depends greatly on the peptide itself. Deciding which peptide to express is crucial since it has been observed that certain peptides placed on the surface of the CP impair viral infectivity or movement through plant tissues, providing low or no VNP production at all (Lico, Capuano et al. 2006).

Tobacco mosaic virus, a tobamovirus, was the first of these viruses used for peptide presentation (Bendahmane, Koo et al. 1999); however, it formed rigid particles, this preventing several modified CPs from assembling, possibly because the subunits were very tightly packed (Smith, Lindbo et al. 2006). This problem can be addressed by using flexuous viruses. Potexviruses have been used for peptide presentation in applications such as vaccinology, drug targeting and delivery, diagnostic kit development and also as an imaging reporter tool (Lico, Benvenuto et al. 2015). However, the use of potyviruses, also flexuous viruses, has gained less attention.

Turnip mosaic virus (TuMV) is a plant virus included in the genus potyvirus. The viral particle has a flexuous tubular structure, approximately 720 nm long and 12-15 nm wide. When presented with a crosslinking agent like glutaraldehyde the virions form nanonets (Cuenca, Mansilla et al. 2016). The use of TuMV as scaffolds has been reported in previous work from our group where a peptide derived from the human vascular endothelial growth factor receptor 3 (VEGFR-3) was successfully expressed on the surface of TuMV VNPs resulting in very high immunogenicity and sensitivity in the detection of specific VEGFR-3 antibodies (Sanchez, Saez et al. 2013). However, this approach is not fitting for every peptide. As a way of being able to produce VNPs with a desired peptide that proves impossible to express due to the aforementioned issues, a different strategy must be attempted, one that does not require any

viral component other than the coat protein itself. Therefore for the expression of these peptides, the option of producing VLPs, instead of virions, seemed a more fitting approach.

This paper is the first report of plant-made VLPs from TuMV as nanoscaffolds for peptide presentation. For the production, the transient expression system based on pEAQ vectors (Sainsbury and Lomonossoff 2008) was used because they allow extremely high-level and rapid production of proteins without viral replication or movement. We undertook the expression of a non-modified CP and of a recombinant CP fused to a peptide derived from the thrombin receptor protein (TR), which participates in the regulation of vascular tone and permeability, as well as muscle growth and bone cell differentiation and proliferation (Martorell, Martínez-González et al. 2008). The TR peptide mimics the effects of thrombin such as platelet aggregation, an increase in tyrosine phosphorylation, inhibition of cAMP, and increase in cytosolic calcium (Landesberg, Burke et al. 2005). Moreover, thrombin is an essential component in the hemostatic, proliferative, and inflammatory responses to injury. This suggests that TR peptide is a promising candidate as a clotting agent for platelet-rich plasma (PRP), hence our interest. The fusion of this peptide to the coat protein of TuMV resulted in a CP 14 amino acids longer than wild type. This peptide impaired virus infection when expressed in virions.

Therefore, in this work we show the feasibility of producing VLPs *in planta* displaying a peptide that hindered the production of virions. In addition, this new biomaterial showed also the ability to log-increase the sensing of antibodies specific to the TR-derived peptide, as shown previously for recombinant infectious virus expressing the VEGFR-3 peptide (Sanchez, Saez et al. 2013).

MATERIALS AND METHODS

1. Construction of chimeric clones

A DNA sequence was synthesized by GeneArt (Regensburg, Germany). The synthetic DNA consisted of *Mlu*I recognition sequence, partial NIb gene (188 nt), the sequence encoding an Alanine (the first amino acid of the CP), sequence encoding the Thrombin Receptor Activating Peptide (TR, aa 42-55, 42 nt) and the sequence encoding a Lysine to complete a *Dra*I restriction site. This DNA sequence was double digested with *Mlu*I and *Dra*I and the fragment of 236 bp purified. The vector (p35Tunos-vec01), derived from an original infectious clone of Turnip mosaic virus (TuMV) was made through the creation of a new unique insertion site for foreign genes between the NIb and the CP genes of the viral genome. NaT constructs are non-aphid transmissible versions of the previous vector, that have a DAE motif in their CP, instead of the natural DAG motif (Touriño, Sánchez et al. 2008). Both vectors were double-digested with *Mlu*I (restriction site being within the gene NIb) and *Nae*I between the first and the second amino acids of the CP, and this fragment was replaced by the *Mlu*I-*Dra*I fragment, resulting in a CP 14 aa longer than wild type (Fig. 1). The maintenance of the recombinants was verified by IC-RT-PCR, followed by sequence analysis.

2. Site-directed mutagenesis

Site-directed mutagenesis of TR-Y11X mutants was performed using p35Tunos-vec01 as a template. The forward primer (ACGCGTGGATGATTGAACAAG) was annealed to the restriction site *Mlu*I. The mutated reverse primers (TR-Y11L: TTAAAAAGGCTC**CA**ACTTATCGTTAG; TR-Y11D: TTAAAAAGGCTCGT**C**CTTATCGTTAG; TR-Y11K: TTAAAAAGGCTC**TTT**CTTATCGTTAGG) were annealed to the restriction site *Dra*I (modified nucleotides are shown in bold and underlined). PCR products were cloned into pCR-Blunt II TOPO-Blunt and confirmed through sequencing. Sequences were obtained from an external sequencing service (SECUGEN, Spain).

3. Plant inoculation and evaluation of genetic stability

Three-week-old *Arabidopsis thaliana*, ecotype RLD, plants were inoculated in rosette leaves with 2 μL per leaf of the plasmid solution at 200 ng/ μL (passage 0). Inocula for subsequent passages (passages 1 and 2) were prepared by grinding infected plants in 50 mM potassium phosphate buffer, pH 7.5, 1:2 (w/v). The crude sap was inoculated with sterile cotton sticks as described (Sánchez, Martínez-Herrera et al. 1998). Tyrosine mutant constructs were inoculated onto three-week-old *N. benthamiana* plants with 5 μL per leaf of the plasmid solution at 200 ng/ μL .

Indirect ELISA tests were carried out in extracts of inoculated *A. thaliana* and *N. benthamiana* plants at 25 days postinoculation, using the monoclonal antibody anti-potyvirus group (anti-POTY) from Agdia, following manufacturer's instructions. The stability of the progeny in the infected plants was assayed by IC-RT-PCR as described (Sánchez, Wang et al. 2003). PCR products were purified (QIAquick, Qiagen, Germany) for subsequent sequencing.

4. Construction of CP and TR-CP expression plasmids

A methionine was added ahead of the CP and TR-CP sequence, as well as *Age*I and *Xho*I restriction sites to flank the fragment. The CP and TR-CP fragments were cloned into p35Tunos-vec01 plasmid and PCR-amplified using Pfu DNA polymerase (Biotools, Spain). PCR fragments were directly cloned into pCRBluntII-TOPO vector (Invitrogen, USA). DH5 α cells were used as the recipient *Escherichia coli* strain for cloning purposes. The clones were plated in Luria-Bertani medium supplemented with 50 $\mu\text{g}/\text{ml}$ kanamycin. Positive clones were later confirmed through sequencing.

Plasmids containing CP or TR-CP were double digested with *AgeI* (Promega, USA) and *XhoI* (Promega, USA). DNA fragments were extracted from 0.8% agarose gels and purified using GeneClean Turbo Kit (MPbio, USA). pEAQ-HT vector, kindly provided by Prof. George Lomonossoff (John Innes Centre, UK), was double digested with the same restriction enzymes and phosphatase-treated to enhance the ligation of the plasmid with the fragments. After purification, fragments and pEAQ-HT were ligated with T4 DNA ligase (New England Biolabs, USA) and later transformed into DH5 α cells and sequenced. The construct was transformed into *Agrobacterium tumefaciens* LBA4404 and propagated at 28°C in Luria–Bertani media containing 50 μ g/mL kanamycin and 50 μ g/mL rifampicin.

5. Expression and purification of VLPs

Transient expression of the CP and TR-CP constructs was done by agroinfiltration into *N. benthamiana* leaves. Plants were grown in a greenhouse maintained at 23–25°C and infiltrated 3–4 weeks after seedling transplantation. *A. tumefaciens* strains were subcultured and grown overnight, pelleted at 2000 $\times g$, resuspended to OD600 = 1.2 in MMA (10 mM MES buffer, pH 5.6; 10 mM magnesium chloride; 450 μ M acetosyringone), and then infiltrated into all the leaves using a blunt-ended 2 mL syringe. Tissue was harvested at 11 days post-agroinfiltration.

VLPs were purified from the *N. benthamiana* (100 g) material as described previously by us for virus preparations (Sanchez, Saez et al. 2013). In summary, plant tissue was finely ground in 0.5 M potassium phosphate buffer pH 7.5, 1:2 (w/v) using an electrical tissue grinder. The resulting solution was extracted with one volume of chloroform at 4°C. Phases were separated by centrifugation and the aqueous phase was filtered through Miracloth. Afterwards, the VLPs were precipitated for 90 min at 4°C with 6% PEG 6000 (w/v) and 4% NaCl (w/v). Particles were recovered by centrifugation for 10 min at 12,000 $\times g$ and then the pellet was resuspended overnight in 0.5 M potassium phosphate pH 7.5, 10 mM EDTA. The solution was clarified by

centrifugation (10 min at $9000 \times g$) and the VLPs pelleted in a Beckman 50.2 Ti rotor (2 h at $80,000 \times g$). The pellet was resuspended in 9 ml 0.25 M potassium phosphate pH 7.5, 10 mM EDTA, and CsCl was added (3.6 g CsCl). The resulting solution was centrifuged at $150,000 \times g$ (Beckman 90 Ti rotor) for 18 h at 4°C . A visible band in the gradient containing the particles was retrieved by extracting it with a syringe and needle. The resulting solution was diluted with 0.25 M potassium phosphate pH 7.5, 10 mM EDTA, the VLPs pelleted by centrifugation in a Beckman 50.2 Ti rotor (2 h at $80,000 \times g$), the pellet resuspended in 50% glycerol (v/v), 10 mM Tris-HCl pH 7.5, 10 mM EDTA and stored at -20°C until further use. VLP concentration was determined using a spectrophotometer considering an absorption coefficient ($A_{0.1\%, 1\text{ cm}}$ at 260 nm) of 2.65 (Brunt 1992). VLP yield was obtained by calculating the amount (μg) of VLP per gram of fresh weight.

6. Immunocapture-reverse transcription-polymerase chain reaction

IC-RT-PCR was performed as described (Nolasco, De Blas et al. 1993) in TuMV virus and VLPs using the monoclonal antibody anti-POTY (Agdia) to capture the virus. The following cycles were used: 3 min 95°C , 35 cycles 30 s 95°C , 30 s 52°C , 1 min 72°C ; 5 min 72°C . Parallel PCR reactions were done with the plasmid (5 ng) used to inoculate plants as template.

Amplification products were analyzed by electrophoresis in 0.8% agarose gel in TAE buffer. Both IC-RT-PCR and PCR products (1067 bp) were digested with *SpeI* and the digestions analyzed in 8% polyacrylamide gel electrophoresis in TBE.

7. RNA agarose gel electrophoresis

TuMV virus and VLPs were added to a mixture of 5M NaCl, SDS 10% and Proteinase K and incubated for 30 minutes at 37°C . Afterwards, a volume of phenol was added to the samples, they were vortexed and centrifuged for 4 minutes at 7000 rpm. The upper phase was

transferred to a clean eppendorf. 2X Loading buffer was added to the samples and a ssRNA ladder (New England BioLabs, USA) and were heated at 65° C for 5 minutes, and later ran in a 1% agarose gel in 1X TBE.

8. SDS-PAGE and Western blot analysis

SDS-PAGE gels (12%) (Mini-PROTEAN TGX gels, Bio-Rad) were run in parallel for the separation of approximately 1 µg of CP VLPs and TR-CP VLPs each. One of the gels was stained with Sypro Ruby Protein Gel Stain (ThermoFisher, USA), while another was transferred to a nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad). Anti-Thrombin Receptor Antibody (ab32611, Abcam, UK) or anti-POTY antibody (Agdia, USA) were used as primary antibody diluted 1:100 in phosphate-buffered saline, pH 7.4, 0.05% (v/v), Tween 20, 2% (w/v) skim milk, followed by the addition of anti-rabbit secondary antibody (AP304A, EMD Millipore, USA) conjugated to alkaline phosphatase, diluted 1:500 in the same buffer. Incubations with the primary and the secondary antibodies were performed at room temperature for 1 h. Membranes were developed with NBT-BCIP.

9. Electron microscopy

Electron microscopy grids (400 Mesh nickel, carbon coated) were precoated with anti-POTY (Agdia, USA) antibody (dilution 1:1000 in 50 mM borate buffer, pH 8.1) by gently placing them, film-down, on top of the antiserum drop and incubated for 10 minutes at room temperature. Grids were then removed with tweezers and rinsed with the same buffer. They were later floated on a 10 µl drop of VLPs (0.05 mg/ml), incubated at room temperature for 15 min, and washed with buffer. Afterwards, the grids were incubated 15 min with anti-Thrombin Receptor Antibody (ab32611, Abcam, UK), diluted 1:20, for decoration. Finally, the grids were

rinsed with distilled water and stained with 2% uranyl acetate for 2 min. Samples were examined on a transmission electron microscope (JEM JEOL 1010, Japan).

10. Immunoassays

An indirect enzyme-linked immunosorbent assay (ELISA) was performed in order to assess the maximum antibody detection sensitivity provided by our VLPs, in comparison with free peptide at equal peptide amounts. Plates (Nunc maxisorp) were coated with 1 µg of CP or 1 µg of TR-CP purified VLPs resuspended in 50 mM sodium carbonate buffer, pH 9.6; or 50 ng of TRAP peptide (S7152, Sigma-Aldrich, USA) dissolved in the same buffer and incubated overnight at 4°C. Plates were washed intensively, and incubated for 2 h with anti-Thrombin Receptor Antibody (Abcam, UK) (different dilutions in phosphate-buffered saline, pH 7.4, 0.05% Tween 20 (v/v), 2% PVP-40 (w/v)). Then, anti-rabbit secondary antibody (AP304A, EMD Millipore, USA) diluted 1:1500 in the same buffer was added and incubated for 1 h at room temperature. Alkaline phosphatase activity was detected after adding *p*-nitrophenylphosphate. The optical density of samples was determined at 405 nm (TECAN Genios Pro, Switzerland).

A dot blot was likewise performed. 10 µl drops of serial dilutions (1:1 – 1:128) of purified VLPs (CP and TR-CP) and the equivalent amount of TR peptide (starting on 1 µg of VLPs and 50 ng of free TR peptide resuspended in 50 mM sodium carbonate buffer, pH 9.6) were placed upon a PVDF blotting membrane (Amersham Hybond-P) at the center of each grid and allowed to dry. The membrane was then blocked with 2% skimmed milk in PBS. Then, the membrane was incubated for 2 h with anti-Thrombin Receptor Antibody (Abcam, UK) diluted 1:100 in phosphate-buffered saline, pH 7.4, 0.05% Tween 20 (v/v), 2% PVP-40 (w/v). Then, the membrane was washed with phosphate-buffered saline, pH 7.4, 0.05% Tween 20 (v/v) buffer. Afterwards, anti-rabbit secondary antibody (AP304A, EMD Millipore, USA) diluted 1:500 in the

same buffer and the membrane was incubated for 1 h at room temperature. Membranes were developed with NBT-BCIP.

RESULTS

1. Production of TuMV VNPs in the form of virions.

Integrity of the constructs was confirmed by sequencing (Fig. 1A and B). Infectivity of the four plasmids was tested by inoculation onto *Arabidopsis thaliana*. In passage 0 (as described in Materials and Methods), only plants inoculated with vec01, vec01-NaT1, and vec01-NaT1-TR (P3, R2) became infected, although only 1 of 52 plants was infected with the last construct (Fig. 1C). Symptoms appeared 9-10 days after inoculation, virus presence in symptomatic tissue was assessed by ELISA, and sequence maintenance in the progeny virus was verified by IC-RT-PCR, followed by sequence analysis. Passage 1 was done with crude sap from vec01, vec01-NaT1 and vec01-NaT1-TR *A. thaliana* infected plants. Only vec01 and vec01-NaT1 crude sap were infectious in this passage. Passage 2 plants were inoculated using crude sap from vec01 and vec01-NaT1 passage 1 plants of the same host. High infectivity was obtained (Fig. 1C), symptoms appearing 6-7 days after inoculation. In both, wild-type and chimera-inoculated plants, symptoms were vein clearing followed by stunting (Martínez-Herrera, Romero et al. 1994).

Fig. 1. Chimeric clones and their infectivity. (A) Schematic illustration of constructs of the genome of the virus indicating the positions of *MluI* and *NaeI* restriction sites for insertion of the foreign epitope. The insertion was performed through the replacement of a *MluI*–*NaeI* fragment containing the epitope sequence. Abbreviations: generic names of potyvirus proteins, 35S promoter from cauliflower mosaic virus (p35S), nos terminator (t). (B) Comparison of the amino acid sequences of Vec01, Vec01-NaT1, Vec01-TR and Vec01-NaT1-TR at the site of epitope insertion in the N-terminal region of TuMV CP. The insertion of the coding sequence for the epitope was between the first and the second amino acid of the TuMV CP (Alanine, Glycine). (C) Infectivity of constructs in *A. thaliana*. Infectivity was assessed by immunocapture-RT-PCR as previously described (Sánchez, Wang et al. 2003)

The very low infectivity of the clones containing the TR epitope prompted us to the construction of three mutants that could restore infectivity by changing the tyrosine in this peptide. This amino acid is not present in the vast majority of CPs of potyviruses so it could be a constraint on virion assembly due to steric hindrances or some other interference with the virus replication cycle. The tyrosine in position 11 of the TR peptide was changed into an acid (D), apolar (L) or basic (K) residue, the resulting mutants being TRY11D, TRY11L and TRY11K respectively (Fig. 2A). In addition, a mutant at position 11 of the vec-01 construct was created

(CPD11Y) where the aspartic acid residue was replaced by a tyrosine to determine if the latter would have an effect on the infectivity of this construct. The position 11 (CPD11Y) mutant, when inoculated on *N. benthamiana* plants, practically abolished the infectivity of the virus (Fig. 2B). However, when the tyrosine (TRY11D, TRY11L and TRY11K) mutants were inoculated, the viral infectivity was not restored (Fig. 2B), implying that the tyrosine might not be the sole cause for the lack of infectivity. Nonetheless, pTR and the tyrosine mutants were inoculated on *Chenopodium quinoa*, a local-lesion host for TuMV (Sánchez, Martínez-Herrera et al. 1998) and the plants showed chlorotic lesions consistent with infection, although not suitable for virus purification. Thus, despite the mutants being infectious in certain hosts they were not capable of infecting a proper host for purification, hence ruling out any possible biotechnological use for the virions.

A			
VECTOR	CONSTRUCT	aa	SEQUENCE NT-CP
p35TunosVec01–TR	pTR	ASFLLRNPNDK	YEPFGETLDAGLTDEQ
p35TunosVec01–TRD	pTRY11D	ASFLLRNPNDK	D E PFGETLDAGLTDEQ
p35TunosVec01–TRK	pTRY11K	ASFLLRNPNDK	K E PFGETLDAGLTDEQ
p35TunosVec01–TRL	pTRY11L	ASFLLRNPNDK	L E PFGETLDAGLTDEQ
p35TunosVec01	-	A	GETLDAGLTDEQ
p35TunosVec01–Y	pCPD11Y	A	GETLDAGLT Y EQ

B	
Inoculum	Infected plants / Inoculated plants (% infected plants)
	<i>N. benthamiana</i>
Mock	0/6 (0%)
pTR	1/6 (17%)
pTRY11D	2/6 (33%)
pTRY11K	0/6 (0%)
pTRY11L	1/6 (17%)
pCPD11Y	1/6 (17%)

Fig. 2. Mutants and their infectivity. (A) Amino acid sequence of constructs pTR, pTRY11D, pTRY11K, pTRY11L, pCPD11Y. (B) Infectivity of mutants in *N. benthamiana*. Infectivity was assessed by immunocapture-RT-PCR as previously described (Sánchez, Wang et al. 2003) . *The *N. benthamiana* plants showing symptoms of virus infections developed necrosis in the inoculated leaves, not systemic infections, making them not viable for purification.

2. Assembly and characterization of TuMV-like particles

1. VLP expression and production

In order to overcome the infectivity problem presented by the TR peptide and to be able to purify these VNPs a new strategy was pursued. CP VLPs and TR-CP VLPs were purified from agroinfiltrated *Nicotiana benthamiana* plants as described in Materials and Methods. The TuMV VLP band was visible in the CsCl gradient and was found in the density range consistent with that of TuMV virions but sparser than the latter.

CP mobility in SDS-PAGE corresponded to the TuMV CP molecular weight of *ca.* 33 kDa and TR-CP showed the slower electrophoretic mobility expected due to having 14 extra amino acids (Fig. 3A). Western analyses showed the reactivity of TR-CP VLP with anti-potyvirus and anti-Thrombin Receptor Antibody (Fig. 3, panels B and C). Another band was also observed below the ~33 kDa band, an artifact present in many potyvirus Western analyses (Sanchez, Saez et al. 2013). The yields obtained are consistent and comparable between virions and recombinant virions in *B. juncea*, and virions and VLPs in *N. benthamiana* (Fig. 3D). JPN1 strain was introduced in this analysis since it is a milder strain than UK1 and is able to infect *N. benthamiana* without promoting necrosis. Therefore, this JPN1 virion production is more comparable to VLP production than UK1 since it is in the same host.

Fig. 3 Production of TuMV virus-like particles in *N. benthamiana* plants. (A) SDS-PAGE analysis of the CP of non-recombinant and TR-recombinant VNPs stained with SyproRuby. (B) and (C) Western-blot analysis with antibodies for potyvirus CP (B), or TR peptide (C). The TR polyclonal antibody only recognized the TR-recombinant TuMV CP (TR-CP). Lane M is a molecular marker, the weights are indicated. The positions of CP and TR-CP proteins are indicated with an arrow. (D) Production yields (µg/g of fresh weight) of several constructs in different hosts.

2. eVLPs

IC-RT-PCR and RNA agarose gel electrophoresis were performed to determine if the VLPs encapsidated RNAs. In both assays we compared TuMV virus and VLPs (Fig.4). In the denaturing agarose gel electrophoresis we observed a band of ~9.3 kb in the virus lane corresponding to TuMV. No band was observed in the VLP lane, neither in that size or below, in case there were smaller RNAs encapsidated. In the IC-RT-PCR we obtained a ~900 bp band in the virus lane, corresponding to the CP, while no band was observed in the VLP lane. This demonstrated that the VLP does not encapsidate RNA of the virus. Both assays were concluding, to the best of our knowledge that the VLPs are empty.

Fig. 4 Electrophoretic analysis of possible RNAs encapsidated within the VLPs. (A) IC-RT-PCR designed to amplify the CP fragment of ≈ 0.9 kb, applied to purified CP-VLPs and TuMV virions. Marker is λ Pst1. (B) RNA agarose gel electrophoresis of phenol-extracted purified CP-VLP and TuMV virions. Marker is ssRNA ladder (New England Biolabs, USA). Arrows point to the CP-related amplicon (A) and the genomic viral RNA (B).

3. VLP visualization

Electron microscope micrographs of CP VLP and TR-CP VLP preparations revealed potyvirus-like long flexuous particles (Fig. 5A, B and C). Although not extensive quantitative comparative measurements were conducted on the images obtained, some preliminary observations can be mentioned. For instance, in several occasions VLPs larger than the virion unit were also found. Also, the grids corresponding to CP VLP and TR-CP VLP preparations showed that CP-VLPs were intact, or almost intact, after incubation at 4, 15 and 28° C. However, TR-CP VLPs seemed affected by some degree of degradation at 28° C (Fig. 5C).

Fig. 5 Transmission electron microscopy of VLPs. VLPs produced by expressing the full-length coat protein of TuMV (CP VLPs; left) and VLPs produced by expressing the full-length coat protein of TuMV with a TR epitope fused to the N-terminal region of the CP (TR-CP VLPs; right). Negatively stained TEM images of CP VLPs and TR-CP VLPs after being incubated at 4° C (A), 15° C (B), or 28° C (C) for 1 h.

3. TR VLPs for very sensitive specific antibody detection

In previous work, purified chimeric virion VNPs showed log increases in their ability to detect antibodies in sera when used as reagents in ELISAs (Sanchez, Saez et al. 2013). Therefore, indirect ELISAs were carried out to assess the specific antibody detection using equivalent amounts of free TR peptide and the TR peptide present in TR VLPs against anti-thrombin receptor antibody. The antibody was serially diluted starting with a 1:25 dilution. We presumed that lower dilutions would give saturated signals in some cases, as indeed was the case (see for instance the first dilutions in Fig. 6A). In TR-CP VLP-coated wells the end-point titre of the commercial antibody was *ca.* thirty-fold greater than in wells coated with equal amounts of free peptide (Fig. 6A). This is indicative that the multimeric presentation of the TR peptide provided by the VLP improved significantly specific antibody detection. This increment in antibody sensing is comparable to the one previously obtained with the VEGFR3 virion VNPs (Sanchez, Saez et al. 2013).

Peptides tend to have low affinity for solid phases (Causey and Dwyer 1996, Yokoo, Togashi et al. 2010, Li, Wu et al. 2011), therefore in order to assess if the free peptide was not binding properly to the plate and therefore giving lower detection values, a dot blot assay was done using a nitrocellulose membrane, with equal amounts of peptide (in a multimeric presentation or as free peptide), serially diluted. Consistent results were obtained (Fig. 6B), since TR-CP VLPs were approximately 20 times more sensitive than free peptide. Consequently this is compatible with the interpretation that multimeric presentation enhances the biotechnologically relevant application of antibody detection.

Fig. 6. Comparative TR antibody detection. (A) Comparative ELISA with free TR peptide and TR VLPs. The black line (threshold) represents the A405 value over which the response of a given solution is considered as positive (three times over background). (B) Comparative Dot Blot of TR-CP VLPs, PepTR and CP VLPs (negative control) at equal amounts.

DISCUSSION

In the present work we report the *in planta* production of VLPs from a potyvirus carrying a heterologous peptide that impairs virus infectivity. Flexuous elongated VNPs (family *Flexiviridae* or *Potyviridae*, for instance) offer some specific traits worth exploring, but have hitherto received less attention compared to viruses with other virion architectures. In the case of potyviruses, few reports are available (Jagadish, Ward et al. 1991, Fernández-Fernández,

Martínez-Torrecuadrada et al. 1998, Kalnciema, Skrastina et al. 2012, Sanchez, Saez et al. 2013). Regarding potyvirus VLPs produced in plants, the coat protein (CP) of potato virus A (PVA) was produced by infecting plants using potato virus X (PVX)-derived vectors (Cеровска, Hoffmeisterova et al. 2008). However, in order to increase PVA CP production it was necessary to double-infect the producing plants with a second virus, potato virus Y (PVY), another potyvirus. Thus, plants used as VLP biofactories were infected with two viruses, complicating their long-term viability as well as their purification. We simplified this by agroinfiltrating plants with high-expression plant vectors directly enabling the recombinant VLP production.

For display, foreign peptides were genetically fused to the N-terminus of the structural viral coat protein (CP) of TuMV. We have previously reported the development of chimeric VNPs containing the VEGFR-3 (Vascular endothelium growth factor receptor-3) (Sanchez, Saez et al. 2013). However, when the same approach was attempted with a thrombin receptor peptide (TR) we found that it severely impaired the ability of the recombinant virus to infect host plants (*A. thaliana* and *N. benthamiana*). Reports indicate that the expression of peptides fused to the CP leads to chimeras with phenotypes and variable yields, due to impaired infectivity (Porta, Spall et al. 2003). For instance, the insertion of just two amino acids right after the first CP amino acid of *Plum pox virus*, another potyvirus severely affected its infectivity (Fernández-Fernández, Martínez-Torrecuadrada et al. 2002). This suggests that the success of the fusion not only depends on the virus itself, but also on the compatibility of the peptide with the assembly mechanisms, viral replication cycle and/or viral movement. Moreover, there is the need to avoid steric hindrances or interference in viral accumulation (Johnson, Lin et al. 1997, Bendahmane, Koo et al. 1999). The peptide's isoelectric point is another critical factor to take into consideration. In PVX, to induce systemic infection, peptides must have a pI value within 5.24-9.18 (Lico, Capuano et al. 2006). Since the TR peptide in this study is of 6.935, pI does not seem to be the cause of the impaired infectivity. Only small peptides shorter than 30 amino

acids have been proposed as amenable to display without interfering with proper assembly, although exceptions to this rule have also been found (Ramqvist, Andreasson et al. 2007). In our case, size did not seem to be the problem since the TR peptide and its mutants consist of just 14 amino acids, well below the 30 amino acid limitation identified by these previous studies. Another potential issue could be the inappropriate folding of displayed peptides due to their amino acid composition and sequence (Murawski, McGinnes et al. 2010). When profiling the amino terminal region of the potyvirus species none of them presented any cysteines or tryptophans, and most did not have tyrosines either. This could suggest that the problem was associated with the TR peptide containing a tyrosine. However, mutants consisting of a change of the tyrosine to other non-aromatic amino acids, in an attempt to restore infectivity, were not successful, and only produced necrosis in the plants (not a systemic infection). This was true in both hosts (*N. benthamiana* and *C. quinoa*), making them inviable for purification. Thus, we cannot conclude that the tyrosine is the sole cause of the impaired infectivity with the TR construct and further studies will be needed to resolve this issue.

To overcome the infectivity problem presented by the TR peptide and obtain recombinant VNPs we pursued a new strategy for the production and purification of biosafe recombinant TuMV VLPs in plants.

The production was attempted in *Nicotiana benthamiana* plants agroinfiltrated with *Agrobacterium tumefaciens* bearing high-expression vectors of the pEAQ family (Sainsbury and Lomonossoff 2008). High level expression of CP and TR-CP were achieved in plant leaves. Despite this, the recovery was still lower than that of virions. When we purified CP VLPs and TR-CP VLPs we obtained approximately 10 μ g/g from *Nicotiana benthamiana*, lower than the approximately 20 μ g/g obtained for virions in the same host. Taking into account that VLPs are non-replicating entities, this yield seems to be quite reasonable. Furthermore, the VLP approach as a whole has its advantages due to the pEAQ vectors, such as being able to produce

and purify them in a 15-18 day time frame, rather than the approximately month and a half normally required when working with infected plants whose infection is started by mechanical inoculation of a few leaves (Spence 1992, Peyret and Lomonossoff 2013).

In order to evaluate VLP assembly and properties, several methods were used. When examined under the electron microscope, the visual inspection of the micrographs confirmed the assembly of CP and TR-CP VLPs. Also, a slight increase in the width of TR-CP VLPs compatible with the expression of the foreign peptide was apparent, although we did not go into a detailed comparison of the parameters defining the particles. In addition, we observed that the overall architecture of TR-CP VLPs retained some of the characteristics of a potyvirus (long flexuous particles, 15-20 nm in width), similar to TuMV wild-type VLPs. However, both CP VLPs and TR-CP VLPs were more heterogeneous in size, displaying particles as small as 100 nm, up to 2000 nm, in contrast with the monodisperse appearance of TuMV virions (Sanchez, Saez et al. 2013). Also VLP thermal stability appeared to be lower in comparison with virions. These differences in comparison with TuMV virions are probably a direct consequence of the lack of nucleic acid in VLPs, an important virion component. Most likely the viral RNA plays a relevant role in the determination of virion length and assembly mechanism. Our work has shown that its presence is not an absolute requirement for particle assembly, but both the assembly process and the final particle physicochemical characteristics are necessarily different in both VNP types.

Numerous studies demonstrate the possibility of recombinant VLPs to bind and encapsidate nucleic acids that are present in the host cells, such as the mRNA of the transgene, ribosomal and/or transport RNAs (Zeltins 2013). However, when assessed by IC-RT-PCR and RNA analysis on agarose gel electrophoresis, we were able to determine, to the best of our knowledge, that the VLPs are empty (Fig.4).

When the novel VLPs were assessed for thermal stability, differences were also observed. Thermal stability has been thoroughly studied for some icosahedral VLPs (Newman, Suk et al. 2003, Ausar, Foubert et al. 2006, Caldeira and Peabody 2011, Schielke, Filter et al. 2011, Zhao, Allen et al. 2012). Reports indicate a correlation in degradation of the particles as temperature increases (Ausar, Foubert et al. 2006). In this study, we observed that TR-CP VLPs started to disassemble at temperatures lower than CP VLPs. Although there are no reports of thermal stability studies for elongated VLPs, when assayed on phage VLPs those displaying foreign peptides denatured at temperatures around 10°C lower than unmodified VLPs (Caldeira and Peabody 2011).

In western blot experiments using the purified particles, TR-CP VLPs were recognized by a commercial antibody against the expressed peptide (Fig. 3), indicating that it maintained its immunological properties when fused to the CP. An additional band was observed below 33 kDa. This band has appeared in previous westerns and was deemed an artifact. Although, this could be due to putative proteolysis and/or premature termination (Jagadish, Ward et al. 1991), no peak was observed when analyzed by MALDI-TOF in previous experiments. In addition, the expression of the peptide did not interfere with the recognition of the VLPs by a potyvirus monoclonal antibody (Jordan and Hammond 1991). The antigen recognized by this mAb is not in the N-terminal region where the peptide was expressed, rather is located in the core region of the viral CP (Jordan 1992). This indicates that the immunological properties of these two different epitopes in the chimeric VLP are not being affected by each other.

The purified TR-CP VLPs showed the ability to log-increase the sensing of antibodies specific to the TR-derived peptide. The VLP nature of being particulate and displaying epitopes on their surface in a dense repetitive array makes them a good means for immunogenicity and specific antibody detection (Peabody, Manifold-Wheeler et al. 2008, Chen and Lai 2013). The ability to increase antibody sensing at the log-level by multimeric presentation of a peptide in a

potyvirus scaffold has already been described by us (Sanchez, Saez et al. 2013), and this technology has led to applied work (Duval, Cruz-Vega et al. 2016), however not for VLPs. Here, we report that these VLPs increased sensitivity at least 30-fold, when compared with equal amounts of free TR peptide. The fact that a substantial increase in the values was obtained when plates were coated with the peptide in multimeric form suggests potential applications for these VLPs in immunochemistry. This increase could be justified by the potyvirus architecture, which consists of approximately 2000 copies of CP displaying the peptide. In addition, some of these chimeric VLPs are much longer than wild-type TuMV, hence gaining sensitivity. The amount of copies per VLP is one of the advantages of flexuous viruses, since in icosahedral VLPs there is a definite and much smaller amount of peptide availability for display.

This work opens the way for further developments based on plant-made flexuous VLPs for nanobiotechnological purposes. These include detection or immunization, as well as being carriers for drug delivery, among other uses. There is still great potential in merging VLPs and plant-expression systems. The full extent of the combination of both technologies needs to be achieved. Our present work constitutes a significant step towards this goal.

Identification of vascular endothelial growth factor receptor-3 autoantibody as a serologic marker of liver fibrosis in bile duct-ligated rats

ABSTRACT

There is a need for new noninvasive biomarkers (NIBMs) able to assess cholestasis and fibrosis in chronic cholestatic liver diseases (CCLDs). Tumorigenesis can arise from CCLDs. Therefore, autoantibodies to tumor-associated antigens (TAA) may be early produced in response to abnormal self-antigen expression caused by cholestatic injury. Vascular endothelial growth factor receptor-3 (VEGFR-3) has TAA potential since it is involved in cholangiocytes and lymphatic vessels proliferations during CCLDs. This study aims to detect autoantibodies directed at VEGFR-3 during bile duct ligation (BDL)-induced cholestatic injury in rat sera and investigate whether they could be associated with traditional markers of liver damage, cholestasis, and fibrosis. An ELISA was performed using VEGFR-3 chimeric viral nanoparticles and VEGFR-3 peptide to detect anti-VEGFR-3 autoantibodies in sera of rats with different degree of liver injury and results were correlated with aminotransferases, total bilirubin, and the relative fibrotic area. Mean absorbances of anti-VEGFR-3 autoantibodies were significantly increased from week one to week five after BDL. The highest correlation was observed with total bilirubin ($R^2 = 0.8450$, $p = 3.04 \times 10^{-12}$). In conclusion, anti-VEGFR-3 autoantibodies are early produced

during BDL-induced cholestatic injury, and they are closely related to cholestasis, suggesting the potential of anti-VEGFR-3 autoantibodies as NIBMs of cholestasis in CCLDs and justifying the need for further investigations in patients with CCLD.

INTRODUCTION

Cholestasis is defined as a decrease in bile flow. It can arise at the hepatocellular level because of impairment of bile secretion by hepatocytes or at cholangiocellular level, generally by obstruction of bile flow through intra- or extrahepatic bile ducts by gall stones or local malignancies. Cholestasis is the pivotal hallmark of the so-called chronic cholestatic liver diseases (CCLDs), but it may also occur in the advanced stage of other chronic liver diseases (CLDs), such as alcoholic liver disease, nonalcoholic fatty liver disease, and chronic hepatitis B and chronic hepatitis C (Jüngst, Berg et al. 2013). When left untreated, cholestasis may drive, in the long term, to tumorigenesis of cholangiocytes (Aller, Arias et al. 2010), the epithelial cells that line bile ducts and normally contribute to the modification of bile volume and composition. This evolution to a malignant phenotype of cholangiocytes, similar to cholangiocarcinoma, takes place through a series of functional and structural changes that affect cholangiocytes, starting early after the initial cholestatic insult by activation, proliferation, and secretion of neuroendocrine factors (Maroni, Haibo et al. 2015). Chronic cholestatic liver injury is also accompanied by the development of hepatic fibrosis referring to the inappropriate tissue repair via excessive connective tissue deposition in the liver (Penz-Österreicher, Österreicher et al. 2011), which is a common scenario CCLDs share with all CLDs. Fibrosis is dynamical as it can progress to cirrhosis, a condition hardly reversible with significant morbidity and mortality and growing prevalence worldwide (Duval, Moreno-Cuevas et al. 2015). Cholestasis and fibrosis have enormous economic impact on health care expenditures which further increase when

cirrhosis and malignant states are reached (Shifeng, Danni et al. 2013). Moreover, current screening methods for cholestasis and fibrosis, especially liver biopsy, have significant limitations (Rockey, Caldwell et al. 2009), thus justifying the exploration of new accurate noninvasive biomarkers (NIBMs) able to early assess cholestasis and fibrosis to estimate the prognosis and determine the surveillance strategies in CCLDs.

Autoantibodies against tumor-associated antigens (TAA) represent promising candidates for NIBMs in liver malignancies, such as cholangiocarcinoma (Mustafa, Nguyen et al. 2016), and early states of malignancies, like early chronic cholestatic liver injury. In some case, the mere presence of autoantibodies to TAA may precede the clinical diagnosis of liver cancer (Himoto, Kuriyama et al. 2005). This offers a window of opportunity to intervene and prevent or redirect the course of the disease. In addition, contrastingly to polypeptides, antibodies do not undergo proteolysis in serum, and therefore they are highly stable with half time in the bloodstream ranging from 7 to 30 days depending on the subclass of immunoglobulin (Hong and Huang 2015). In cholangiocarcinoma patients, autoantibodies directed against p53, heat shock protein 70, enolase 1, and ribonuclease/angiogenin inhibitor 1 have already been reported (Limpaiboon, Sripa et al. 2004, Rucksaken, Pairojkul et al. 2014). The mechanism that triggers the autoantibody response against TAA has still not been elucidated but could be consequent to abnormal self-antigen expression by tumor cells, through chemical alteration, mutation, posttranslational modification, misfolding, aberrant cleavage or localization, and overexposure and/or exposure or spillage of new TAA, in conjunction with the development of an inflammatory reaction within the tumor microenvironment (Lleo 2014, Hong and Huang 2015). The elicited autoantibodies oriented to these neo-epitopes may be involved in tumor surveillance and regulation, a process that involves activation of immunocompetent cells, leading to tumor cell apoptosis (Lleo 2014). Nowadays, thanks to the progress in the knowledge of CCLDs, in part through the development of animal models like the bile duct ligation (BDL)

model of chronic cholestatic liver injury, new autoantibodies to TAA with potential as NIBM can be discovered.

Even though to date only autoantibodies to vascular endothelial growth factor receptor-2 have been reported in a glioblastoma patient (Iizuka, Komiyama et al. 2014), all the proteins from the vascular endothelial growth factor (VEGF) family are potential TAA because of their crucial role in tumor growth. Vascular endothelial growth factor receptor-3 (VEGFR-3), a tyrosine kinase receptor for VEGF-C and VEGF-D, has been shown to play a critical role in the pathogenesis of different proliferative events during CLDs, including that of lymphatic vessels (Tugues, Morales-Ruiz et al. 2005, Chung and Iwakiri 2013) and cholangiocytes (Gaudio, Barbaro et al. 2006, Maroni, Haibo et al. 2015). In the latter, the upregulation of VEGFR-3 and secretion of VEGF-C ligand have appeared to mediate the adaptive proliferative response of cholangiocyte to BDL-induced early cholestatic liver injury via an autocrine mechanism that involves activation of inositol.

Virus-derived nanoparticles (VNPs), in the form of virions or virion-like particles (VLPs), have been used as scaffolds to support the display of antigens genetically or chemically conjugated to structural virus proteins. The advantage of these biological nanoparticles for this purpose is that VNPs are highly ordered, multimeric in nature, and of nanometric dimensions, all of them features highly desirable to prime good immunological responses (Jennings and Bachmann 2008). This paper used VEGFR-3 chimeric VNPs derived from *Turnip mosaic virus* (TuMV), a potyvirus. The ability to detect specific VEGFR-3 antibodies using the VNPs has already been assessed (Sanchez, Saez et al. 2013). Moreover, VEGFR-3 VNPs increase antibody-sensing, compared to free VEGFR-3 peptide. Therefore, in this work, we want to determine if the detection of autoantibodies of VEGFR-3 is enhanced by the use of these VNPs.

MATERIALS AND METHODS

1. Animals

Thirty-six male Wistar rats (300–400 g) were included in this study and divided into seven groups. Five rats were used as a control group without operation. Sixteen underwent BDL. These animals were sacrificed one week later ($n = 5$), three weeks later ($n = 7$), and five weeks later ($n = 5$). Fifteen rats underwent sham operation. These animals (per group) were sacrificed at same times as BDL groups. 191 All rats were housed in plastic cages and maintained in an environmentally controlled room ($22 \pm 2^{\circ}\text{C}$, $65 \pm 10\%$ humidity) with a 12-hour light/dark cycle; food and water were given ad libitum. The Institutional Committee on care and use of Experimental Animals at the Tecnológico de Monterrey approved this study with protocol number 2013-Re-001.

2. Bile duct ligation

All rats that underwent BDL and sham operations received intraperitoneal injection of ketamine at 50 mg/kg (Anesket, Pisa Agropecuaria, Hidalgo, Mexico) and xylazine at 10 mg/kg (Sedaject, Vedilab, DF, Mexico) to induce anesthesia. In BDL groups, a laparotomy was performed, and the common bile duct was double-ligated with 4-0 silk and cut between the ligatures. In rats that underwent sham operation, common bile duct was isolated but not ligated, neither cut. Then linea alba and skin were closed with 4-0 Vicryl and 3-0 Prolene, respectively. All surgeries were performed under aseptic conditions. Before sacrificing, rats were anesthetized by ether inhalation (J. T. Baker, Center Valley, PA, USA) and blood samples were collected by cardiac puncture. Serum was obtained and stored at -20°C . Then, animals were immediately sacrificed by cervical dislocation, and the liver was removed, sectioned, and fixed in 10% neutral buffered formalin for morphometric quantification of fibrosis.

3. Serum biomarkers of liver injury and cholestasis

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TB) were determined spectrophotometrically using an automatic biochemical analyzer ILab Aries (Instrumental Laboratory, Holliston, MA, USA).

4. Morphometric quantification of fibrosis

Postfixed liver tissues were embedded in paraffin. Liver slices 4 µm thick were prepared and stained with picrosirius red stain kit (Abcam, Cambridge, MA, USA) according to manufacturer's protocol. Collagen content was assessed by morphometric analysis of picrosirius red-stained liver sections. Thirty pictures per animal were taken at 10x magnification with a microscope (Olympus BX41 TF, Melville, NY, USA) and digital camera (Olympus DP20, Melville, NY, USA). Pictures were processed using imaging software (Image J 1.49v, National Institutes of Health, USA). Data were expressed as relative fibrotic area.

5. Production of VEGFR-3 chimeric viral nanoparticles (VNPs)

These VNPs were produced as described in Sánchez et al., 2013. In summary, a synthetic DNA consisting of the MluI recognition sequence, partial NIb gene (188 nt), sequence encoding the first amino acid of the TuMV CP (Alanine), sequence encoding the VEGFR-3 peptide (Vascular Endothelial Growth Factor Receptor, VEGFR-3, aa 1279–1298, 60 nt) and StuI restriction site was double-digested with MluI and StuI and purified. Vectors p35Tunosvec01 (vec01) and p35Tunos-vec01-Nat1 (vec01-Nat1) (Tourino et al., 2008) were double-digested with MluI (restriction site within the NIb gene) and NaeI (the midst of NIb and CP) and the 190 bp fragment replaced by the 250 bp MluI–StuI fragment. The result was a TuMV CP 20 aa longer than wild type. Plasmids from constructs pVER3- and pVER3-Nat were purified. Three-week-old *Arabidopsis thaliana* plants, ecotype RLD, were inoculated with plasmid DNA (passage 0).

Plasmid DNA was inoculated onto two leaves per plant (2 μ L per leaf, of a plasmid solution at 200 ng/L). Inocula for subsequent passages (passages 1 and 2) were prepared by grinding infected plants in 50 mM phosphate buffer, pH 7.5. The crude sap was inoculated with sterile cotton sticks as described (Sánchez et al., 1998). Passage 2 was to *A. thaliana* and Indian mustard (*Brassica juncea*) plants.

VNPs were purified either from Arabidopsis (15 g) or Indian mustard (150 g) infected material as described (Spence, 1992). Briefly, plant tissue was finely ground in 0.5 M potassium phosphate pH 7.5, 1:2 (w/v) in an electrical tissue grinder, at 4 °C. The resulting suspension was extracted with one volume of chloroform at 4 °C. Phases were separated by centrifugation; aqueous phase was filtered through Miracloth. After that, virus particles were precipitated with 6% PEG 6000 (w/v), 4% NaCl (w/v). They were allowed to precipitate for 90 min at 4 °C. The particles were recovered by centrifugation for 10 min at 12,000 $\times$ g. The pellet was resuspended overnight in 0.5 M potassium phosphate pH 7.5, 10 mM EDTA. The solution was clarified by centrifugation (10 min at 9000 $\times$ g) and the virus pelleted (2 h at 80,000 $\times$ g). The pellet was resuspended in 0.25 M potassium phosphate pH 7.5, 10 mM EDTA, and CsCl was added (3.6 g CsCl to 9 ml of solution). The resulting solution was subjected to centrifugation at 150,000 $\times$ g for 18 h at 4 °C. A visible band in the gradient containing the particles was recovered by punching the tube with syringe and needle. It was diluted in 0.25 M potassium phosphate pH 7.5, 10 mM EDTA, the virus pelleted by centrifugation (2 h at 80,000 $\times$ g), the pellet resuspended in 50% glycerol (v/v), 5 mM Tris pH 7.5, 5 mM EDTA and stored at -20 °C until further use. VNP concentration was determined spectrophotometrically considering an absorption coefficient ($A_{0.1\%}$, 1 cm at 260 nm) of 2.65.

6. Indirect enzyme-linked immunosorbent assay (ELISA)

An indirect ELISA was performed to detect the presence of VEGFR-3 specific autoantibodies in rat sera from control, sham, and BDL groups. Plates (Nunc MaxiSorp, San Diego, CA, USA)

were coated with 0.03 µg of VEGFR-3 peptide (LIPPSO, Girona, Spain) diluted in 50 mM sodium carbonate buffer pH 9.6 and incubated overnight at 4°C. After blocking overnight at 4°C with 3% skimmed milk, plates were incubated 2 h at 30°C with rat sera diluted in PBS with 0.05% Tween 20. Then, horseradish peroxidase-conjugated rabbit anti-rat (Abcam, Cambridge, MA, USA) diluted 1 : 5000 was added and incubated for 45 min at 30°C. Horseradish peroxidase activity was assessed by adding 1-Step ABTS Substrate Solution (Life Technologies, Waltham, MA, USA). Optical density of samples was determined at 405 nm (Tecan Genios Pro, Männedorf, Switzerland). Negative and positive controls were included under the same conditions. Each sample was tested as duplicates.

A second indirect ELISA was performed to determine the autoantibody-sensing of VEGFR-3 comparing VEGFR-3 peptide to VEGFR-3 chimeric VNPs. Plates (Nunc MaxiSorp, San Diego, CA, USA) were coated with equivalent amounts of VEGFR-3 peptide (0.5 µg of VEGFR-3 peptide and 0.03 µg of VEGFR-3 chimeric VNPs) diluted in 50 mM sodium carbonate buffer pH 9.6 and incubated overnight at 4°C. The following steps were done as stated in the previous ELISA.

RESULTS

1. Validation of the BDL animal model

A panel of traditional markers of BDL-induced liver injury (AST and ALT), cholestasis (TB), and fibrosis (relative fibrotic area) was assessed in order to validate the BDL animal model (Table 1). As expected, liver/body weight ratio, AST, ALT, TB, and the relative fibrotic area were all significantly elevated in the three BDL groups when compared to control group and remained significantly higher than equivalent sham groups throughout the experiment. Although a tendency of increment according to the time of BDL is observed for all the studied markers during the five weeks of the experiment, only differences in the relative fibrotic area

between all consecutive groups and in the liver/body weight ratio between the 1st week and the 3rd week reached significance. Furthermore, no difference was significant between the control and sham groups for all the studied markers. Therefore, our BDL animal model was validated as an accurate model of liver injury, cholestasis, and fibrosis.

Table 1. Measurements of traditional markers of liver injury and cholestasis in serum, and fibrosis in the liver.

	Control (n = 5)	Week 1 (n = 5)	Sham Week 3 (n = 5)	Week 5 (n = 5)	Week 1 (n = 5)	BDL Week 3 (n = 7)	Week 5 (n = 4)
Liver/body weight ratio (%)	3.98 ± 0.32	3.87 ± 0.47 ^b	4.07 ± 0.17 ^b	3.68 ± 0.32 ^b	5.35 ± 0.40 ^a	6.99 ± 1.02 ^{ac}	6.84 ± 0.65 ^a
AST (U/L)	141.20 ± 13.93	160.20 ± 37.16 ^b	117.40 ± 22.07 ^b	126.20 ± 23.79 ^b	862.83 ± 394.96 ^a	974.71 ± 330.69 ^a	1084 ± 607.64 ^a
ALT (U/L)	71.60 ± 13.05	71.80 ± 14.86 ^b	67.60 ± 13.13 ^b	72.20 ± 18.30 ^b	172.83 ± 69.08 ^a	139.57 ± 49.43 ^a	176.25 ± 81.89 ^a
TB (mg/dL)	0.12 ± 0.05	0.10 ± 00.00 ^b	0.10 ± 00.00 ^b	0.08 ± 0.05 ^b	7.62 ± 2.68 ^a	8.97 ± 1.77 ^a	8.95 ± 2.32 ^a
Relative fibrotic area (%)	0.4292 ± 0.1239	0.45 ± 0.15 ^b	0.50 ± 0.05 ^b	0.45 ± 0.11 ^b	0.84 ± 0.19 ^a	8.33 ± 1.20 ^{ac}	17.17 ± 0.33 ^{ac}

Data were expressed as mean ± standard deviation. ^a*P* < 0.05 for BDL and sham groups versus control group; ^b*P* < 0.05 for BDL groups versus equivalent sham groups; and ^c*P* < 0.05 for BDL 195 at week 3 versus BDL at week 1 and BDL at week 5 versus BDL at week 3. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BDL, bile duct ligation; TB, total bilirubin.

2. Serum autoantibodies to VEGFR-3 are produced during BDL-induced chronic cholestatic liver injury

The analysis of indirect ELISA showed that, in every BDL group, the mean of absorbances associated with the serum levels of autoantibodies to VEGFR-3 is significantly higher than control and equivalent sham groups (Fig. 1). At the 1st week, the mean of absorbances increased up to 4 times compared to the control group (mean value of absorbance at the 1st week after BDL: 0.5103 ± 0.0814; *P* = 1.69e - 06). The mean of absorbances continued to increase at the 3rd week (mean value of absorbance at the 3rd week: 0.5352 ± 0.0935) and reached a maximum level at the 5th week (mean value of absorbance at the 5th week: 0.5864 ± 0.1307) in BDL groups. Nevertheless, no significant difference between consecutive BDL groups

was reached. Means of absorbances associated with the serum levels of autoantibodies to VEGFR-3 of sham groups was not significantly different from that of the control group.

Fig. 1. Comparative evaluation of the absorbance relative to the serum levels of autoantibodies to VEGFR-3 by indirect ELISA. Optical density was measured at 405 nm in control group (white bar) (n = 3), BDL groups (black bars) at week 1 (n = 5), week 3 (n = 7), and week 5 (n = 4), and sham groups (grey bars) at week 1 (n = 3), week 3 (n = 3), and week 5 (n = 3). Data were expressed as mean $\pm$ standard deviation. BDL: bile duct ligation.

The results of the comparative ELISA with VEGFR-3 peptide and VEGFR-3 VNPs coating was consistent with the previous results (Fig. 2). The mean of absorbances associated with the serum levels of autoantibodies to VEGFR-3 peptide corresponds to the previous figure. However, an increase in absorbances was observed for those detected with VEGFR-3 VNPs. Instead of just a 4-fold increase to control in the first week with VEGFR-3 peptide, a 10-fold increase is observed by detection with VEGFR-3 VNPs. Absorbances continued to increase week by week.

Fig. 2. Comparative evaluation of the absorbance relative to the serum levels of autoantibodies to VEGFR-3 by indirect ELISA. Optical density was measured at 405 nm in control group (white bar) (n = 3), BDL groups detected with VEGFR-3 VNPs (black bars) at week 1 (n = 5), week 3 (n = 7), and week 5 (n = 4), and sham groups (light grey bars) at week 1 (n = 3), week 3 (n = 3), and week 5 (n = 3). BDL groups detected with VEGFR-3 peptide (dark grey bars) at week 1 (n = 5), week 3 (n = 7), and week 5 (n = 4), and sham groups (light grey bars) at week 1 (n = 3), week 3 (n = 3), and week 5 (n = 3). Data were expressed as mean $\pm$ standard deviation. BDL: bile duct ligation.

3. Absorbances associated with the serum levels of autoantibodies to VEGFR-3 are correlated to AST, ALT, TB, and the relative fibrotic area

Correlations between absorbances associated with the serum levels of autoantibodies to VEGFR-3 from the indirect ELISA and AST (Figure 3(a)), ALT (Figure 3(b)), TB (Figure 3(c)), and the relative fibrotic area (Figure 3(d)). The highest correlation was observed between absorbances associated with the serum levels of autoantibodies to VEGFR-3 and TB ($R^2 = 0.8450$, $P = 3.04e - 12$). Absorbances associated with the serum levels of autoantibodies to VEGFR-3 also closely correlated with AST ($R^2 = 0.5865$, $P = 1.24e - 06$), ALT ($R^2 = 0.4555$, $P = 4.90e - 05$), and the relative fibrotic area ($R^2 = 0.4442$, $P = 6.47e - 05$).

Fig. 3. Correlations between absorbances associated with the serum levels of autoantibodies to VEGFR-3 and a panel of traditional markers of liver injury, cholestasis, and fibrosis measured in serum and liver. Correlations between absorbances associated with the serum levels of VEGFR-3 autoantibody obtained in the indirect ELISA and (a) serum AST activity, (b) serum ALT activity, (c) TB serum levels, and (d) relative fibrotic area in liver obtained in all experimental groups. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; TB, total bilirubin; VEGFR-3, vascular endothelial growth factor receptor-3.

DISCUSSION

Autoantibodies are not considered anymore as an exclusive hallmark of autoimmune disorders. In fact, autoantibodies are abundant and ubiquitous in the serum of all humans (Nagele, Han et al. 2013). Using protein microarrays, Nagele EP et al., 2013, demonstrated that age, gender, and disease influence autoantibodies serum level in human and proposed that disease-induced perturbations in individual autoantibody profiles present an opportunity to

detect accurately and diagnose diseases (Nagele, Han et al. 2013). In CLDs, autoantibodies have been detected first in autoimmune liver diseases, such as primary biliary cirrhosis and primary sclerosing cholangitis, the clinically most important CCLDs, and more recently in viral hepatitis, drug-induced hepatitis, alcoholic liver disease, nonalcoholic fatty liver disease, hepatocellular carcinoma, and cholangiocarcinoma (Himoto and Nishioka 2013). Autoantibodies detected in CLDs target a wide repertoire of host antigens, which include proteins from the nucleus, the cytoskeleton, liver kidney microsomes, the mitochondria, and the cytoplasm of neutrophilic granulocytes, as well as liver soluble antigens, asialoglycoprotein receptors, TAA, and stress proteins (Himoto and Nishioka 2013). However, this list is continuously growing thanks to the advances in the understanding of the pathogenesis of CLDs and the improvement of conventional technologies (ELISA, indirect immunofluorescence, and immunoblotting) for the detection of autoantibodies as well as the development of new efficient technologies (laser bead immunoassays, antigen microarrays, and line immunoassays), which offer the opportunity to manage a higher number of samples with lower costs.

In the present study, we focused on autoantibodies directed against TAA since cholestasis and CCLDs have been associated with liver and biliary tree malignancies, such as cholangiocarcinoma and hepatocellular carcinoma. Hepatocellular carcinoma may arise in the long term of chronic cholestatic liver injury. However, this causal relationship seems unusual as only 1–12% of patients with hepatocellular carcinoma manifest obstructive jaundice as the initial complaint (Qin and Tang 2003). Cholestasis with chronic inflammation has also been linked to carcinogenesis in cholangiocarcinoma. In combination, both conditions can promote the four major cancer phenotypes: (1) autonomous cell proliferation; (2) invasion/metastases; (3) escape from senescence; and (4) evasion of cell death (Blechacz and Gores). Moreover, it has been demonstrated that malignant cholangiocytes proliferate more rapidly after BDL, thereby emphasizing the role of obstructive cholestasis as a potent promotor of intrahepatic

cholangiocarcinoma growth and progression. The mechanism of autoantibody induction against TAA is still an area of debate. Autoantibodies are probably induced because cells undergoing malignant transformations express self-antigens that were not evident before (Lleo 2014). Another explanation is that, during the earliest stage of tumorigenesis, proteins are likely mutated, overexpressed, posttranslationally modified, misfolded, aberrantly cleaved, or aberrantly localized in tumor cells (Hong and Huang 2015). Under these conditions, altered proteins may pass from an unreactive state to TAA, thus eliciting the production of autoantibodies to try to control the growth of the TAA-expressing tumor.

In particular, we focused on VEGFR-3, the specific receptor for VEGF-C and VEGF-D, because (1) it is already known that VEGF-C/VEGF-D/VEGFR-3 signaling pathway is crucial for the growth and maintenance of tumors, including cholangiocarcinoma (Fava, Demorrow et al. 2009, Leyva-Illades, McMillin et al. 2012) and hepatocellular carcinoma (Jia, Zhuang et al. 2009); (2) VEGFR-3 is involved in cell proliferation during chronic cholestatic liver injury (Gaudio, Barbaro et al. 2006); (3) VEGFR-3 expression is altered in response to chronic cholestatic liver injury (Gaudio, Barbaro et al. 2006, Franchitto, Onori et al. 2013); and (4) structural and functional changes occur in VEGFR-3-expressing cells during chronic cholestatic liver injury (Yamauchi, Michitaka et al. 1998, Maroni, Haibo et al. 2015). In response to obstructive cholestatic liver injury, cholangiocytes evolve according to three interrelated phenotypes (Maroni, Haibo et al. 2015). Early after BDL, an ischemic-reperfusion phenotype, characterized by cholangiocyte depolarization, abnormal ion transport, hypometabolism, and hepatocytic apoptosis, is induced (Maroni, Haibo et al. 2015). This phenotype, in turn, could trigger the activation of cholangiocytes which start to proliferate and secrete neuroendocrine factors to compensate for the loss of biliary cells and sustain secretory activities (Aller, Arias et al. 2010, Maroni, Haibo et al. 2015). In the long term, cholestatic injury drives to tumorigenesis, where the tumorous tissue principally consists of cholangiocyte parenchyma (Maroni, Haibo et al. 2015). A study of

Gaudio et al. revealed that this adaptive proliferative response of cholangiocyte to cholestasis is mainly mediated by the upregulation of VEGFR-3 and secretion of VEGF-C, through activation of inositol 1,4,5-triphosphate/[Ca²⁺]_i/protein kinase C- α , and phosphorylation of Src/extracellular signal-regulated kinases 1/2, during the first week after BDL injury (Gaudio, Barbaro et al. 2006). The increased proliferation of cholangiocytes in normal rats chronically treated with recombinant VEGF-C and the decreased cholangiocyte proliferation when VEGF is immunoneutralized by antibodies to VEGF-C sustain this theory (Gaudio, Barbaro et al. 2006). Along with cholangiocyte proliferation, VEGF-D/VEGFR-3 signaling pathway has also been implicated in the pathologic reactivation of lymphangiogenesis during CLDs (Chung and Iwakiri 2013). In patients with viral forms of CLDs, the numbers of lymphatic vessels and their areas are increased according to the degree of fibrosis (Yamauchi, Michitaka et al. 1998). Therefore, VEGFR-3 may be converted in TAA during the early steps of the progression of CCLDs towards tumorigenesis. For that reason, we investigated the production of autoantibodies to VEGFR-3 in the BDL-model of chronic cholestatic liver injury.

To determine whether BDL induces a measurable production of VEGFR-3 autoantibodies in serum, we performed an indirect ELISA by coating wells with VEGFR-3 peptide (Sanchez, Saez et al. 2013). We found that absorbances associated with the serum levels of autoantibodies to VEGFR-3 in BDL groups were systematically higher than control rats. This result demonstrates that BDL triggers the serum production of autoantibodies to VEGFR-3, which is consistent with the study of Thomas et al., 2012, who reported that the lack of lymphatic drainage, as occurs in the BDL animal model, promotes autoimmunity. Absorbances associated with the serum levels of autoantibodies to VEGFR-3 in BDL groups increased significantly as soon as the 1st week after BDL when compared to control group. This may offer the opportunity of using autoantibodies to VEGFR-3 to early diagnose BDL-induced chronic cholestatic liver injury. Moreover, we showed that these absorbances kept on increasing throughout the five weeks of

the experiment even though no significance was reached between consecutive BDL groups, thus suggesting that autoantibodies to VEGFR-3 could be appropriate to monitor the progression of BDL-induced chronic cholestatic liver injury.

Studies on the identification of new NIBMs have revealed that autoantibodies to TAA represent promising candidates (Rucksaken, Pairojkul et al. 2014). For example, it has been demonstrated that plasma levels of IgG autoantibodies against heat shock protein 70 are accurate to diagnose cholangiocarcinoma and that specificity increases by adding autoantibodies to enolase-1 and ribonuclease/angiogenin inhibitor 1 (Rucksaken, Pairojkul et al. 2014). Performing correlations between the serum levels of a potential biomarker and traditional markers of CLD pathologic events is a valid strategy for the study of new NIBMs (Veidal, Vassiliadis et al. 2010). Therefore, to give the first evidence about the potential of autoantibodies to VEGFR-3 as NIBMs of BDL-induced liver injury, cholestasis, and fibrosis, we performed correlations between absorbances associated with the serum levels of autoantibodies to VEGFR-3, obtained in the ELISA, and a panel of traditional markers of liver injury (AST and ALT), cholestasis (TB), and fibrosis (relative fibrotic area). AST and ALT are NIBMs that reflect hepatic injury, providing valuable information regarding the overall hepatic function (Gowda, Desai et al. 2009). The analysis of aminotransferase serum activity is commonly used as a first step in the evaluation of patients with suspected CLD along with the determination of other serum components. Bilirubin is a marker of cholestasis (Mesa, De Vos et al. 1997). A TB test is generally ordered to help diagnose CCLD, such as PSC and PBC. Moreover, hyperbilirubinemia, which characterizes severe CCLDs, has been recognized as a major predictor of clinical outcomes in both PBC and PSC (Shapiro, Smith et al. 1979, Helzberg, Petersen et al. 1987). The relative fibrotic area, obtained by computer-assisted image analysis of picrosirius red-stained liver sections, is representative of the liver deposition of collagen I and collagen III, two of the main components of the extracellular matrix. Progressive accumulation

of collagen proteins occurs during liver fibrosis progression (Bataller and Brenner 2005). Therefore, the relative fibrotic area can be used to distinguish the different stages of liver fibrosis (Yoshioka 2013). In liver diseases, excessive collagen deposition leads to structural disruption of the normal liver architecture, liver stiffness, portal hypertension, and eventually hepatic failure (Reynaert, Thompson et al. 2002, Mori, Fujii et al. 2011, Iredale, Thompson et al. 2013). In consequence, methods to supervise the progression of fibrosis are crucial to implement treatment strategies to prevent such events. In this study, we showed that correlations between absorbances relative to the serum levels of autoantibodies to VEGFR-3 and AST, ALT, TB, and the relative fibrotic area were all significant. Also, by using VEGFR-3 VNPs to detect autoantibodies, sensitivity was increased. This is attractive because some autoantibodies levels in serum are very low at the beginning of the disease and might be difficult to detect. Hence creating more sensitive detection agents like chimeric VNPs could be of great use.

These results prove that autoantibodies to VEGFR-3 are correlated to BDL-induced liver injury, cholestasis, and fibrosis and support the need to further study these autoantibodies in more types of animal models of liver injury, such as the carbon tetrachloride model, as well as in human subjects with CCLD. Interestingly, the correlation with TB was the highest with R^2 of 0.845. This finding, in parallel to the observation that the mean value of absorbances associated with the serum levels of autoantibodies to VEGFR-3 is increased directly after BDL and stays high without enabling the distinction between one, three, and five weeks of BDL, a common tendency to TB but not to the relative fibrotic area, suggests that levels of autoantibodies to VEGFR-3 may be better associated with cholestasis than fibrosis. This provides the first evidence on the potential application of autoantibodies to VEGFR-3 as NIBMs of cholestasis in CCLDs. To confirm it, further studies on human patients with CCLD are needed.

CONCLUSIONS

This in vivo study demonstrated that BDL promotes the production of VEGFR-3 specific autoantibodies that are early measurable in the serum as soon as the first week after BDL-induced chronic cholestatic liver injury. Although serum levels of autoantibodies to VEGFR-3 significantly correlated with markers of liver damage and fibrosis, the highest correlation was with TB, suggesting that autoantibodies to VEGFR-3 are closely related to BDL-induced cholestasis.

Part 2. Chemical functionalization

Chemical conjugation of peptides and low-molecular-weight molecules on the surface of Turnip mosaic virus nanoparticles

ABSTRACT

Nanobiotechnology is a science emerging nowadays. The use of nanoparticles for different applications like drug delivery, antibody-sensing, image enhancers or biosensors are thoroughly studied. Many different types of biomaterials are employed for this purpose. In this work, the use of plant-made *Turnip mosaic virus*-derived nanoparticles is presented. These viral nanoparticles (VNPs) present a sole cysteine that is reactive and accessible, therefore making it attractive for bioconjugation. A vasoactive intestinal peptide (VIP) and several low-molecular-weight compounds, including an antitumoral organometallic compound were conjugated to the TuMV VNPs cysteine residue. This work presents the groundwork for multifunctionalization of VNPs for countless applications.

INTRODUCTION

Over the last decade or so, the use of viral nanoparticles for biotechnological applications has emerged for a myriad of applications in multiple fields. Most applications have been developed around viruses with a spherical symmetry, although some important ones have also been carried out on helicoidal symmetry ones. Within plant viruses, tobamoviruses (TMV) and potexviruses (mostly PVX) are the helicoidal-symmetry viruses most often used in the field (Wen, Rambhia et al. 2013, Lico, Benvenuto et al. 2015, Steele, Peyret et al. 2017). Within this type of viruses, those with an elongated flexuous architecture, such as PVX or PapMV are

particularly interesting for a number of reasons, such as a very high number of identical coat protein (CP) subunits for peptide and protein display, a high aspect ratio, or a better performance in tumor homing and tissue penetration (Lico, Capuano et al. 2006, Steinmetz, Mertens et al. 2010, Shukla, Ablack et al. 2013) in addition to the flexuosity trait. Somewhat surprisingly, members of the Potyvirus genus of plant viruses have not received comparable attention, and publications about them in this context are scarcer (Jagadish, Ward et al. 1991, Fernández-Fernández, Martínez-Torrecuadrada et al. 1998, Kalnciema, Skrastina et al. 2012, Sanchez, Saez et al. 2013). Potyvirus particles share with potexviruses the elongated flexuous architecture, being even longer which can be advantageous for some applications. Their structure has not been determined by crystal analysis but a notable level of structural knowledge is available for them (Wang and Stubbs 1994, Baratova, Efimov et al. 2001, Kendall, McDonald et al. 2008, Yang, Wang et al. 2012, Agirrezabala, Mendez-Lopez et al. 2015). These studies have revealed a common architecture for flexible plant viruses. The filaments are 120-130 Å in diameter, both the N- and C-terminal residues of potyviruses are thought to be surface located, and the CPs are arranged following helical symmetry with slightly less than 9 sub-units per turn (Gerald, Amy et al. 2008).

Turnip mosaic virus (TuMV) is a flexuous filamentous plant virus belonging to the Potyvirus genus. The virions of ≈ 700 nm long and 12-15 nm in diameter are made up of ≈ 2000 identical copies of ≈ 33 kDa coat protein (CP) subunits that cover a molecule of viral RNA of ≈ 10 kb in size. TuMV has a great homology in amino acid sequence to the coat proteins of other potyviruses, the greatest differences found in the N-terminal region. Despite this, little is known about their amino acid disposition, as well as the structural conformation of the coat protein subunit. Several conserved sites are found within the potyviruses sequences, being the WCIN motif, located in the coat protein (CP) region of the potyvirus genome, the 12th most conserved

site among the 17 sites (Zheng, Wayper et al. 2008). Each CP contains at least one cysteine residue that could play a role in structural stability or chemical conjugation.

Targeting thiols, amines or other functional groups in viral capsids have enabled the coupling of small molecules to the CP (Wang, Chan et al. 2003, Lewis, Destito et al. 2006, Soto, Blum et al. 2006). In the majority of cases, chemical functionalization has been carried out on amino acid residues located at surface of the CP. In the case of several tobamoviruses, their sole cysteine is not available (due to its position in the structure) and cysteine residues had to be incorporated to the surface for chemical conjugation (Royston, Ghosh et al. 2008, Koch, Wabbel et al. 2015). Until now, potyviruses have only been modified genetically to expose epitopes on their surface (Gonzalez 2011, Kalnciema, Skrastina et al. 2012, Sanchez, Saez et al. 2013), but no chemical conjugation has been reported. Therefore, the use of the natural cysteine residue present in the capsid for this purpose was attempted.

In this work we determine the availability and reactivity of the cysteine residue in the CP to be used for chemical conjugation of a hapten-like molecule, a low-molecular-weight compound and a peptide. Furthermore, we propose a model for amino acid disposition and structural conformation of TuMV CPs. Our aim is to show the potential of TuMV particles for chemical functionalization using its natural cysteine, without the need of any genetic modification of the CP. This functionalization can enhance the VNPs properties and tailor them to specific applications like antibody sensing, drug delivery and tumor bombardment.

MATERIALS AND METHODS

1. TuMV VNP production

Turnip mosaic virus, isolate UK1 was propagated in plants of Indian mustard (*Brassica juncea*) and harvested 30 days post-inoculation. For VLPs, transient expression of the CP

constructs was done by agroinfiltrating *N. benthamiana* leaves 3–4 weeks after seedling transplantation. *Agrobacterium tumefaciens* strains were subcultured and grown overnight, pelleted at $2000 \times g$, resuspended to OD600 = 1.2 in MMA (10 mM MES buffer, pH 5.6; 10 mM magnesium chloride; 450 μ M acetosyringone), and then infiltrated into all the leaves using a blunt-ended 2 mL syringe. Tissue was harvested at 11 days post-agroinfiltration.

Virions and VLPs were purified from *B. juncea* and *N. benthamiana*, respectively. Plant tissue was finely ground in 0.5 M potassium phosphate buffer pH 7.5, 1:2 (w/v) using an electrical tissue grinder. The resulting solution was extracted with one volume of chloroform at 4°C. Phases were then separated by centrifugation and the aqueous phase was filtered through Miracloth. Afterwards, the VNPs were precipitated for 90 min at 4°C with 6% PEG 6000 (w/v) and 4% NaCl (w/v). Particles were then recovered by centrifugation for 10 min at $12,000 \times g$ and the pellet was resuspended overnight in 0.5 M potassium phosphate pH 7.5, 10 mM EDTA. The solution was clarified by centrifugation (10 min at $9000 \times g$) and the VNPs were pelleted in a Beckman 50.2 Ti rotor (2 h at $80,000 \times g$). The pellet was resuspended in 9 ml 0.25 M potassium phosphate pH 7.5, 10 mM EDTA, and 3.6 g CsCl was added. The resulting solution was centrifuged at $150,000 \times g$ in a Beckman 90 Ti rotor for 18 h at 4°C. A visible band in the gradient containing the particles was retrieved by extracting it with a syringe and needle. The resulting solution was diluted with 0.25 M potassium phosphate pH 7.5, 10 mM EDTA. Afterwards, the VNPs were pelleted by centrifugation in a Beckman 50.2 Ti rotor (2 h at $80,000 \times g$), the pellet was resuspended in 50% glycerol (v/v), 10 mM Tris-HCl pH 7.5, 10 mM EDTA and stored at -20°C until further use.

VNP concentration was determined using a spectrophotometer considering an absorption coefficient ($A_{0.1\%, 1\text{ cm}}$ at 260 nm) of 2.65 (Brunt 1992). VNP yield was obtained by calculating the amount (μg) of VNP per gram of fresh weight.

2. Transmission electron microscopy

Grids (400 nickel mesh, carbon coated) were placed on one drop of the conjugated VNPs (wt, biotin-conjugated and vip-conjugated) and incubated at room temperature for 15 min. They were washed with 50 mM borate buffer pH 8.1, incubated 15 min with anti-POTY antibody (AGDIA), anti-biotin antibody (Thermofisher) or anti-VIP antibody (XXX), respectively and diluted 1:20 for decoration. Then, the grids were washed with distilled water and stained with 2% uranyl acetate. Samples were examined on a transmission electron microscope (JEM JEOL 1010).

3. Dithiothreitol (DTT) and Iodoacetamide (IAA) treatment

First, observe the spectrum of the TuMV VNPs using 1 μ l (0.5 μ g/ μ l) of the purified particles at a Nanodrop spectrophotometer. Afterwards, expose 10 μ l of the TuMV VNPs to 2 μ l of 10 mM DTT. Incubate at 37 °C for 30 minutes. Take an aliquot and observe in Nanodrop. Add 1 μ l of 125 mM iodoacetamide to the sample. Incubate ON at 37 °C, without light. Take an aliquot and observe in Nanodrop. The experiment was done in triplicate, one of the samples was only treated with IAA, other with both DTT and IAA and a negative control that was not treated.

4. Cysteine-alkylation with I-AEDANS

The alkylating reagent, I-AEDANS, was used to determine if the TuMV CP has solvent-exposed sulfhydryl groups. The alkylation reaction was initiated by addition of 10 μ L of a 10 mM I-AEDANS solution to 600 ng of virus or VLP followed by incubation at room temperature. The alkylation reaction was terminated after 5 minutes by the addition of 10 μ L of a 1.0 M DTT solution to the samples (the same experiment was done after 30 minutes or 2 hours of incubation). The samples were then loaded onto an SDS-PAGE gel to separate the individual

protein bands and to remove excess I-AEDANS. Protein alkylation was visualized by UV illumination of the PAGE gel prior to staining with Coomassie brilliant blue.

5. *Turnip mosaic virus* model

Protein structure predictors using a variety of approaches that included homology modeling, fold recognition, hybrid methods and even ab initio procedures failed to provide a 3D model incorporating a sufficient number of residues and that were consistent with the scarce available evidence on the structure of the CP of TuMV or related potyviruses. This evidence was essentially the following: (1) a qualitative, schematic model (no atom coordinates) of the structure of the potato virus A (PVA) coat protein derived from the experimental data obtained by tritium bombardment combined with predictions of secondary structure (Baratova, Efimov et al. 2001), and (2) a low resolution structure (no atomic details neither) of the soybean mosaic virus (SMV) experimentally obtained by combining fiber diffraction, cryo-electron microscopy, and scanning transmission electron microscopy (Gerald, Amy et al. 2008, Kendall, McDonald et al. 2008, Agirrezabala, Mendez-Lopez et al. 2015).

Given the high sequence identity among the CPs of PVA, SMV, and TuMV and the existing structural information on PVA and SMV, we obtained a 3D atomic model of the coat protein of TuMV as follows. (i) We used the sequence of PVA to firstly assemble in silico the secondary structure elements identified by the model of the CP of this virus (Baratova, Efimov et al. 2001), and (ii) then we combined these elements to assemble the tertiary structure suggested by this qualitative model for PVA. These two tasks were accomplished in a modular way by using the modeling and optimizing resources of the software Chimera. The resulting 3D full model of the CP of PVA was minimized, equilibrated, and re-optimized in the presence of water in molecular dynamics calculations with the CHARMM force field using the NAMD software. Finally, this 3D model was refined with ModRefiner. (iii) Taking this refined model of the PVA CP as template,

3D model structures of the CPs of SMV and TuMV were obtained using the SwissModel modelling services in project mode. These 3D models that incorporated atom coordinates for SMV and TuMV coat proteins were next subjected to the same optimization-refinement process applied to the PVA protein. (iv) By applying the helical symmetry of the low-resolution structure of the SMV particle (Gerald, Amy et al. 2008), a model of the assembly consisting of nine copies of the 3D model atomic structure of the CP subunit by helical turn was prepared with Chimera for SMV. (v) Finally, the assembly model for the TuMV particle was obtained by structural superposition of CPs of SMV and TuMV and application of the same helical symmetry of the SMV particle.

6. Biotin conjugation

For the chemical conjugation of the EZ-Link™ Iodoacetyl-PEG2-Biotin to TuMV virions, 2 mg of Iodoacetyl-PEG2-Biotin were dissolved in 1ml of ultrapure water. Afterwards we diluted it 1:20 to get a 0.1 µg/ul working solution. Then, 28 µl of the Iodoacetyl Biotin solution were added to 7 µl of TuMV virions (0.1 µg/ul) resulting in 4-fold excess of Iodoacetyl Biotin Reagent. The reaction was mixed and incubated in the dark for 90 minutes at RT.

7. Western Blot analysis

Proteins were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad). The membrane was then blocked with 2% skimmed milk in PBS. CP detection (wt, biotin-conjugated or VIP-conjugated) was carried out after incubation with the primary monoclonal antibody (anti-POTY (Agdia) diluted 1:500, anti-biotin (ThermoFisher) diluted 1:500 or anti-VIP (GeneTex) diluted 1:1000, respectively) in phosphate-buffered saline, pH 7.4, 0.05% (v/v), Tween 20, 2% (w/v) skim milk; followed by binding the secondary antibody anti-mouse (Agdia) conjugated to alkaline phosphatase diluted

1:200 (wt, biotin-conjugated) or anti-rabbit conjugated to AP diluted 1:1500. Incubations with the primary and the secondary antibodies were performed at room temperature for 1 h. Membranes were developed with NBT-BCIP.

8. Enzyme-linked immunosorbent assay (ELISA)

An indirect enzyme-linked immunosorbent assay (ELISA) was performed in order to assess the maximum antibody detection sensitivity provided by our VNPs, in comparison with free peptide at equal peptide amounts. Plates (Nunc maxisorp) were coated with 0.25 µg of TuMV virions or 1.25 µg of biotin-conjugated TuMV virions resuspended in 50 mM sodium carbonate buffer, pH 9.6; or 1 µg of EZ-Link™ Iodoacetyl-PEG2-Biotin (Thermofisher) dissolved in the same buffer and incubated overnight at 4°C. Plates were washed intensively, and incubated for 2 h with anti-biotin antibody (Thermofisher) (different dilutions in phosphate-buffered saline, pH 7.4, 0.05% Tween 20 (v/v), 2% PVP-40 (w/v)). Then, anti-mouse secondary antibody (AGDIA) diluted 1:200 in the same buffer was added and incubated for 1 h at room temperature. Alkaline phosphatase activity was detected after adding *p*-nitrophenylphosphate. The optical density of samples was determined at 405 nm (TECAN Genios Pro, Switzerland).

9. Vasoactive intestinal peptide (VIP) conjugation

VIP peptide (HSDAVFTDNYTRLRKQMAVKKYLNSILN) was conjugated to VNPs via the heterobifunctional cross-linking reagent Sulfo-SIAB (Thermofisher). First, 1.7 mg of Sulfo-SIAB was dissolved in 1 ml of ultrapure water. The diluted 1:50 for working solution (it must be protected from light). In a sterile Eppendorf put 10 µl of ultrapure water. Then add 7 µl of the crosslinker solution and 7 µl of VIP (0.1 µg/µl), and let it react for 3 hours at RT without light. Afterwards, add 7 µl of the VNPs to the VIP-crosslinker reaction, resulting in a 10-fold excess of

VIP and 30-fold excess of crosslinker against the VNP. Leave the reaction ON at RT without light. For mock samples, the same protocol applies without adding the 7 μ l of the crosslinker.

RESULTS AND DISCUSSION

An alignment of 49 sequences of different species of the potyvirus genus was done in order to determine the conserved sites and observe the presence of cysteines. The WCIEN motif, located in the coat protein (CP) region of the potyvirus genome, was ranked the 12th most conserved site among the 17 sites (Zheng, Wayper et al. 2008). Of all the potyviruses analyzed only Canna Yellow Streak Virus (CaYSV) virus didn't have this motif, but either a WAIEN motif. Surprised by the presence of only one cysteine in the whole CP, we decided to study the assembling and reactivity of this cysteine for possible conjugations.

Due to the key interactions that cysteine residues usually have in the structural conformation of protein, we performed assays to observe the importance of the sole cysteine in this matter. At first, we aimed to evaluate the assembling capacity by doing a point mutation in the CP, replacing the cysteine by a serine. Electron micrographs from both TuMV virions and VLPs show very similar characteristics in length and appearance. Therefore, we did the mutation in the VLP form and wondered if its self-assembling capacity could be accomplished despite this mutation. We produced the chimeric VLPs (and wt VLPs for control) in agroinfiltrated *Nicotiana benthamiana* plants and purified them, obtaining similar yields for both. Afterwards, we proceeded to make grids with the purified chimeric VLPs for their assessment by electron microscopy. We observed flexuous elongated particles, similar as those from wt VLPs, but with a difference in width and length (Fig.1). Those from the mutated particles were much thinner and shorter than the wt. Also, fewer particles were observed. This could mean that, although the cysteine is not essential for the self-assembling capacity, it does

have a role in the conformation and structure of the VNP, as well as hindering the production of the VLPs.

Fig. 1. Self-assembling of TuMV VLPs. Chimeric VLPs, cysteine mutant (C replaced by S at position 140 of CP).

Which could be some of the major interactions occurring in the TuMV CP was the question in consideration. Two cysteines in close proximity will form a disulfide bond, significantly stabilizing a tertiary structure. However, since only one cysteine is present, we speculated if this residue was actually part of a dimer, bonding to the cysteine of another CP. In order to prove this, TuMV virions were purified from infected *Brassica juncea* plants, as described in materials and methods, yielding 70 µg of pure TuMV per 1 g infected leaves. The purity of TuMV was determined by a single protein band at 33 kDa by SDS-PAGE, which is the expected size of TuMV coat protein. These samples were observed at the spectrophotometer before and after being exposed to dithiothreitol (DTT) and iodoacetamide (IAA) (Fig. 2). Reduction and alkylation of cysteine residues using DTT and IAA, respectively, will cleave disulfide bonds and prevent disulfide bond reformation. We expected changes that could only be attributed to the presence of cysteine, and its reduced state. However, no major changes were observed. The pattern of UV absorbance was very similar before and after the treatments, suggesting that no dimers are present. Therefore, the question relied on the accessibility and reactivity of this residue, and its use for conjugation.

Fig. 2 Virus UV absorbance pattern. Spectrums obtained before and after treatments with DTT and IAA for 30 min and 24 h.

The alkylating reagent, I-AEDANS, was used to determine if the TuMV CP had exposed sulfhydryl groups. Protein alkylation was visualized by UV illumination of an SDS-PAGE gel prior to staining with Coomassie Brilliant Blue (Fig. 3A). After 5 or 30 minutes, or even 2 hour incubation, it was evident that the TuMV samples (virions and VLPs), as well as the positive control (BSA, 35 cysteine residues) had reacted with I-AEDANS. By UV illumination we could observe the ~33 kDa band expected for the TuMV CP (Fig. 3B). However, it is interesting to mention, that a tobamovirus (Pepper mild mottle virus) was also used in this assay as a control since it also has one cysteine. Nonetheless, in either treatment could we find a band in the expected 17 kDa range. This result made us infer that the flexuous nature of our potyvirus might be responsible for this accessibility.

Fig. 3 Reactivity of cysteine in TuMV CP. 12% SDS-PAGE with samples treated with I-AEDANS for 30 minutes. A) Coomassie stained gel; B) UV illumination of the same gel.

Since TuMV has not been crystallized as TMV, the only way for us to do a more thorough assessment of the difference in accessibility and further reactivity of the cysteine residue was through a model of the TuMV CP. Given the procedure followed to construct the 3D model of the TuMV CP (see Material and Methods), the assembly structure of the viral particle (Fig. 4) has to be regarded as merely tentative. Nonetheless, we expected that the model could provide at least qualitative information on the spatial arrangement of different sequence segments, especially with regard to accessibility and separation between outer and inner structural domains.

Fig. 4. TuMV 3D model. A) Ribbon diagram of a wheel representing the assembly of nine subunits of the coat protein by helical turn in the TuMV particle. B) Ribbon diagram of the atomic 3D structural model of the TuMV coat protein.

We can observe differences between both viruses, for example in the length (300 nm for TMV vs. ~700 nm for TuMV). However, the diameter and number of CPs are quite similar in both viruses, inferring that tobamoviruses are packed more tightly. Also, in regards with the subunits per turn, potyviruses seem to have more space between each CP. This could be a reason for the flexibility of the potyviruses, as well as the accessibility of the cysteine residue. That although it appears to be in a similar position as the one in the tobamovirus, the possibility of reaching it is easier. This could also explain the previous result of I-AEDANS not being able to conjugate to the cysteine in PMMV. This is consistent with previous work with tobamoviruses, where a cysteine codon was inserted within the N-terminus of the CP ORF (McCormick and Palmer 2008), since the sole cysteine present was not accessible.

After determining the reactivity of the cysteine residue in the TuMV, we proceeded to conjugate small compounds. Due to the good results obtained with I-AEDANS, the chemistry for conjugation was decided to remain the same. Therefore, EZ-Link™ Iodoacetyl-PEG2-Biotin (ThermoFisher), a mid-length, haloacetyl activated linker was chosen due to it being sulfhydryl-reactive with the cysteine in the TuMV CP, as well as having a biotin molecule in the other end (Fig. 5).

Fig. 5. Schematic representation of biotin-conjugated TuMV CP. CP model (white); N-terminus of the TuMV VNP (pink); cysteine (yellow); iodoacetyl group (blue); biotin molecule (green).

The biotin-linker was coupled to the external surface of TuMV virion and VLP and the molar ratio of EZ-Link™ Iodoacetyl-PEG2-Biotin to TuMV was 4:1. The optimal cross-linker concentration and incubation time to conjugate the biotin-linker to TuMV were determined by Western blot. Successful conjugation of biotin-linker-TuMVCP was assessed by SDS-PAGE and Western blotting (Fig. 6A). As expected, our staining showed ~33 kDa band, consisting of our TuMV CP and biotin-linker-TuMVCP. The Western blot analysis of nitrocellulose membrane exposed to anti-poty antibody showed a band in both the conjugated and non-conjugated TuMVCP. In addition, the conjugation of the hapten-like molecule (biotin) did not interfere with the recognition of the virus by a potyvirus monoclonal antibody (Jordan and Hammond 1991). The antigen recognized by this mAb is not hindered by the conjugation, although it is located in the core region of the viral CP (Jordan 1992) where accessibility might have been compromised. Likewise, an anti-biotin antibody Western was performed and showed a protein band in the lane of the conjugation (biotin-linker-TuMVCP) with a molecular weight of ~33 kDa and no band where no conjugation was done.

Another important aspect of our conjugation strategy was to maintain the integrity of the TuMV particles. To investigate the nanoparticle structure of our conjugate, we applied transmission electron microscopy. TEM images showed that the filamentous shape of TuMV particles remained intact after the conjugation (Fig. 6b). We observed that the overall architecture of the biotin-linker-TuMVCP conjugates retained the characteristics of a potyvirus (long flexuous particles, 15-20 nm in width), overall similar to TuMV wild-type VNPs. Immunosorbent electron microscopy was also performed (as described in Materials and Methods) and biotin particles can be observed in the surface of the TuMV after decorated with the anti-biotin antibody (Fig. 6B).

Fig.6. Chemical conjugation to the cysteine. A) SDS-PAGE and Western with anti-poty antibody and anti-biotin antibody. *stands for biotin-conjugated; VNP: Viral nanoparticle; B) Electron microscopy with and without immunodecoration (anti-biotin antibody). TEM: Transmission electron microscopy; ISEM: Immunosorbent electron microscopy (antibody coating).

Another approach to assess the conjugation was by enzyme-linked immunosorbent assay (ELISA). Our ELISA results proved the conjugation between TuMVCP and biotin. Also, the biotin-linker-TuMVCPs showed the ability to log-increase the sensing of antibodies specific to biotin. The nature of the potyvirus particles of being particulate and displaying the hapten-like compound (biotin) on their surface in a dense repetitive array makes them a good means for

immunogenicity and specific antibody detection (Peabody, Manifold-Wheeler et al. 2008, Chen and Lai 2013). The ability to increase antibody sensing at the log-level by multimeric presentation of a peptide in a potyvirus scaffold has already been described by us (Sanchez, Saez et al. 2013), and this technology has been transferred into applied work, for autoantibody-sensing (Duval, Cruz-Vega et al. 2016). However, all these were obtained from a genetic modification approach, not by chemical conjugation. Here, we report that the biotin conjugated virus increased sensitivity at least 15-fold, when compared with equal amounts of free biotin (Fig. 7). This increase could be justified by the potyvirus architecture, which consists of approximately 2000 copies of CP displaying biotin. The fact that a substantial increase in the values was obtained when plates were coated with the hapten-like compound in multimeric form suggests potential applications for these conjugated nanoparticles in immunochemistry.

Fig. 7. Biotin antibody detection. Comparative ELISA with free biotin and biotin-conjugated TuMV virions. The black line (threshold) represents the A405 value over which the response of a given solution is considered as positive (three times over background).

After proving the conjugation of small compounds, we decided to use the technology with a molecule with specific applications. The era of targeted therapies has emerged to reduce the cost of treatment and minimize unwanted side effects. Nanotechnology offers novel

opportunities for disease treatment in human health care because they can be tailored as wanted, with different cargoes and targets. Many viral nanoparticles have been used for this purpose. Recent studies indicated that filamentous plant VNP have enhanced tumor homing and tissue penetration, as well as superior pharmacokinetic properties compared to isometric VNP (Geng, Dalhaimer et al. 2007, Shukla, Ablack et al. 2013). This is mainly due to increased surface of the filamentous particle compared to icosahedral particles. The larger surface area of filamentous VNP provides more potential binding sites, compared to isometric VNP thus increasing targeting capacity and specificity (Steinmetz, Mertens et al. 2010). Hence, flexuous nanoparticles have been drawing more attention recently as targeted imaging and therapeutic agents, although the most studied are still icosahedral VNP. To assess the possibility of conjugating a compound with anti-tumor activity, an organometallic compound was selected.

The selected compound is Iridium (III), an anticancer complex used in drug delivery or tumour bombardment (Liu, Habtemariam et al. 2011, Liu and Sadler 2014). The conjugation was performed and preliminary TEM micrographs were obtained. In comparison with the control, non-conjugated TuMV VNP (Fig. 8A), the iridium conjugated (Fig. 8B) show a similarity in morphology (flexuous cylinder). However, the overnight conjugated particles display “round-shaped” masses, consistent with the electrodense iridium, confirming the conjugation.

Fig. 8. Transmission electron microscopy of anti-tumor compound. A) wt TuMV particles; B) Iridium (III)-conjugated VNP after ON reaction.

Finally, in order to further functionalize our VNP and provide it with a wider spectrum of applications, we decided to attempt the conjugation of a peptide to the cysteine residue. Chemical conjugation could provide a useful extension of surface display besides the more obvious genetic approach. Moreover, surface presentation of foreign polypeptides introduced genetically into the capsid is usually restricted to approximately 20 amino acids (Johnson, Lin et al. 1997, Chatterji, Burns et al. 2003, Porta, Spall et al. 2003). Therefore, this approach could expand the possibility of bigger peptides or proteins.

For this we investigated several cross-linkers in the primary steps to find the optimal one. An amine- and sulfhydryl-reactive heterobifunctional cross-linker was chosen: Sulfo-SIAB (sulfosuccinimidyl[4-iodoacetyl]aminobenzoate). NHS esters react with primary amino groups (-NH₂) present on the side chain of lysine (K) residues and the N-terminus polypeptides; while iodoacetyl groups react with the free sulfhydryls by nucleophilic substitution of iodine with a thiol group resulting in a stable thioether linkage. This cross-linker was chosen due to having a similar chemistry as the one with biotin. Both have an iodoacetyl group, which worked well in the previous conjugation, therefore we chose to maintain this chemistry as the linking strategy.

We selected the vasoactive intestinal peptide (VIP), a neuropeptide described as a potent anti-inflammatory factor and also related with autoimmune diseases. It is a good candidate based on its amino acid composition (HSDAVFTDNYTRLRKQMAVKKYLNSILN) since in only 28 amino acids it has three lysine residues, favoring the conjugation (besides the N-terminus) and no cysteines that could directly attach covalently to the VNP (making it a good control for the linker efficiency). Moreover, this is a peptide that had been problematic to express in the surface of either virion or VLP by a genetic approach, and a chemical functionalization might be the way to overcome these problems. Our first attempt was to produce infectious virus, expressing the VIP peptide in the N-terminus of the TuMV CP. However, the chimeric construct was not obtained. Because capsid function can play an important role in virus infectivity, we

next attempted to express the chimeras as VLPs, produced in *Nicotiana benthamiana* plants. This happened before in our group and we were able to obtain chimeric VLPs, when a peptide hindered the infectivity of the virions. Nonetheless, in this case, the chimeric construct did not expressed properly in planta, so no VLPs were purified. From these results we concluded that the introduction of this peptide sequence probably interfered with capsid assembly or production. Therefore, a chemical conjugation could allow this peptide to be exposed in the surface of either virion or VLP, without any of the complications mentioned above.

We managed to conjugate the VIP peptide to TuMV VNPs with the help of the Sulfo-SIAB linker. To confirm the conjugation, an SDS-PAGE and a Western blot were performed. In figure 9A, corresponding to conjugated virion, we can see bands consistent to the 33 kDa CP in the three cases: chemical conjugation and the two negative controls. In the western blot using antibodies against the CP and VIP, we saw that all the bands reacted with the anti-TuMV antibody, but just the one with linker and peptide is VIP positive. The same results were obtained with VLPs (Fig. 9B). We can also observe that several bands above the 33 kDa bands appear in the VIP conjugation lane. This is consistent with the molecular weight increase when conjugating one, two or three VIP peptides per CP. This might be occurring because the linker is attaching itself to the VIP peptide through the lysine residues also, allowing another peptide to be linked through the haloacetyl part of the linker. Although the reactivity of haloacetyl-activated supports toward sulfhydryls is relatively selective, these can react with methionine, histidine, or tyrosine under appropriate conditions (Hermanson, 2013).

Fig. 9. Conjugation of VIP peptide to TuMV VNPs. (A) SDS-PAGE and Western Blot for non-conjugated and conjugated (*) virions. (B) SDS-PAGE and Western Blot for non-conjugated and conjugated (*) VLPs.

Either by genetic or chemical functionalization, VNPs from *Turnip mosaic virus* can be modified for different applications. The next approach could be the combination of both conjugation strategies in order to create multi-functional VNPs.

4. GENERAL DISCUSSION

From a material point of view, *Turnip mosaic virus* is a promising candidate for nanobiotechnological applications. TuMV, as other elongated viruses, is assembled from identical coat proteins to generate a highly homogeneous nanostructure with a flexible filamentous shape (Le, Lee et al. 2017). Being a plant virus, TuMV does not represent a biological hazard because of its non-infectivity towards other organisms. Nonetheless, we have demonstrated that it is also possible to obtain TuMV virus-like particles (VLPs) devoid of nucleic acid due to the in vitro self-assembly mechanisms of CP monomers. The production of these nanomaterials (either virions or VLPs) can be easily generated using plants as biofactories (through infection or agroinfiltration) and in the latter case, scaled up through molecular farming with some commercial facilities already established, such as Medicago (D'Aoust, Couture et al. 2010) or Bioproducts (Charudattan and Hiebert 2007). However, in our case, it is still necessary to optimize each step in order to improve VLP production and downstream processing of plants, in order to recover the highest possible number of VLPs.

Throughout this thesis, we have presented that TuMV VLPs are amenable to genetic and chemical modification thus providing an array of synthetic and biological approaches to produce a library of nanoparticles with distinct functionality. The direction of this work has gone from the genetic modification of TuMV virions; through the generation of novel TuMV empty VLPs, and the later functionalization of said VLPs; onto chemical conjugation through the natural cysteine residue in the TuMV CP.

For peptide display, icosahedral plant viruses have always been the obvious choice (Sainsbury, Canizares et al. 2010). Yet, one of the advantages of elongated viruses, such as tobamoviruses, potexviruses and our potyviruses, is that their capsids contain a large number of coat protein copies with helical symmetry, making the system very attractive for peptide display (McCormick and Palmer 2008). Reports are found in literature regarding the genetic modification of flexuous viruses, including potyviruses (Babin, Majeau et al. 2013, Sanchez, Saez

et al. 2013, Shukla, Ablack et al. 2013, Lee, Uhde-Holzem et al. 2014, Dickmeis, Honickel et al. 2015, Lico, Benvenuto et al. 2015). Although TMV, a tobamovirus, was the first example of peptide presentation of elongated viruses, it forms rigid particles and several modified CP were not competent for virion assembly because subunits were very tightly packed (Smith, Lindbo et al. 2006). Therefore, the use of flexuous elongated nanoparticles, like TuMV seemed like a good idea as scaffolds. Yet despite these characteristics the application of these virus particles is often limited by problems associated with viral replication and recombination which hinder the desired function (Pokorski and Steinmetz 2011).

We encountered that difficulty throughout the development of these nanoparticles. Although a peptide derived from the human vascular endothelial growth factor receptor 3 (VEGFR-3) was successfully expressed on the surface of the TuMV virion (Sanchez, Saez et al. 2013) (Sanchez, Saez et al. 2013), this approach proved not fitting for every peptide. The foreign peptide interfered with the infectivity of the virus. To circumvent these limitations we used high expression vectors (pEAQ vectors) that take advantage of a modified leader sequence from CPMV to induce hypertranslation of cognate mRNAs (Sainsbury and Lomonossoff 2008) facilitating the production of chimeric virus-like particles that assemble in the absence of the viral genome. With this approach we eliminated the need of an infection competent virus while decreasing time of production and costs. And it worked. We were able to obtain chimeric TuMV VLPs (genetically fused with a thrombin receptor peptide).

These TuMV VNPs enhanced antibody sensing through the genetic modification of TuMV virions or VLPs by 30-fold against free peptide. This enhancement was further proved in a liver fibrosis mice model. First, by the use of our immunoassay (with a VEGFR3 peptide coating) autoantibodies detection (Duval, Cruz-Vega et al. 2016); and later by using our chimeric VNPs to increase the sensitivity of the ELISA by 10-fold against the control. This allowed an earlier detection of the disease, in correlation with other non-invasive biomarkers.

Another important characteristic of our VLPs is that they were empty VLPs (eVLPs), with no encapsulated nucleic acid. This is a very significant quality in biomedical applications, for example when used as enzymatic reaction vessels (Comellas-Aragones, Engelkamp et al. 2007) or cancer therapeutics (Sanchez-Sanchez, Cadena-Nava et al. 2014, Zdanowicz and Chroboczek 2016, Rohovie, Nagasawa et al. 2017), among others.

After the generation of these novel nanoparticles, the challenge had now become to be able to display peptides that hinder the production of TuMV VLPs, as well as bigger peptides or other molecules. In order to do this we had to change the functionalization strategy: chemical conjugation. Up until that point, the only type of chemical functionalization tried with TuMV was by using glutaraldehyde to create nanonets displaying immobilized *Candida antarctica* lipase B (Cuenca, Mansilla et al. 2016). Individual CP conjugation of TuMV had not been attempted before this work.

A bigger understanding of the potyviral architecture was needed to face this task. The greatest problem is that the potyvirus particle structure has not yet been crystallized, although a notable level of structural knowledge is available for them (Wang and Stubbs 1994, Baratova, Efimov et al. 2001, Gutiérrez, Bastos-Aristizábal et al. 2011, Yang, Wang et al. 2012, Agirrezabala, Mendez-Lopez et al. 2015). Potyviral CPs are divided into three domains. CryoEM, fiber diffraction and immunological analysis show that N- and C-termini are exposed on particle surface (Gerald, Amy et al. 2008, McDonald, Kendall et al. 2010, Stubbs, Kendall et al. 2012). The more conserved core or central domain is facing towards the inside (Urcuqui-Inchima, Haenni et al. 2001).

In order to make a TuMV CP structure and have more information to determine the right conjugation strategy, computational modelling was pursued. Using other potyvirus or potexvirus models and through calculus of molecular dynamics, the first TuMV CP model was obtained. With the help of this model, we observed the structural conformation of the amino

acids in the TuMV CP and were able to determine the residues that could be assayed for conjugation. Because of its free sulfhydryl group, the cysteine residue in the CP seemed like a good choice. It would readily and spontaneously form disulfide bonds with other sulfhydryl-containing ligands under oxidative conditions; or form thioether linkages by alkylation using maleimide or haloacetyl molecules (Poole 2015, Henkel, Röckendorf et al. 2016). However, according to the model, the sole cysteine of the CP was in the central domain, which appeared difficult to reach for conjugation. Nonetheless, it had to be attempted since a free cysteine rarely is available in a CP's surface and if it is reactive it would constitute an excellent choice for chemoselective modification (Fodje and Al-Karadaghi 2002). Also, chemically conjugating via the available cysteine residues in the TuMV CP would allow approximately 2000 low-molecular-weight molecules or peptides per particle.

The alkylating-fluorescent reagent, I-AEDANS, was used to determine if the TuMV CP had exposed sulfhydryl groups. The iodoacetyl group would react to the cysteine's sulfhydryl group and after loaded in a protein gel, a band in the 33 kDa range (correspondent to TuMV CP) should appear. The reaction was performed with different incubating times, in case the reaction is slow, or the availability of the cysteine is scarce. Nonetheless, in the smallest reaction time (5 minutes) we were able to observe the expected bands for both TuMV VNPs. As a control, a tobamovirus (*Pepper mild mottle virus*) was assayed, since tobamos also have just one cysteine. When comparing the TuMV CP model and the TMV structure, we saw that they have the cysteine in a very similar position. Nevertheless, the tobamovirus did not react with the I-AEDANS, even at the highest incubation time. This is consistent with the different chemical conjugation approaches made in tobamos. Cysteine residues had to be introduced by genetic engineering, primarily in the N-terminus of their CP in order for them to be surface-exposed, and therefore available for conjugation (Yi, Nisar et al. 2005, Royston, Ghosh et al. 2008, Geiger, Eber et al. 2013, Koch, Wabbel et al. 2015).

After proving the reactivity of the TuMV, another compound was chosen. The possibility of confirming the conjugation was sought; therefore a biotin molecule with a PEG2 linker and an iodoacetyl group was selected. We wanted to determine the conjugation by immunoassays. A SDS-PAGE and Western Blot were performed. It showed the conjugation by displaying a band near the 33 kDa mark when assayed with an anti-biotin antibody. Since we wanted to see if the chemical conjugation to TuMV also enhanced the antibody sensing capacity, an ELISA was performed, comparing free biotin against biotin-conjugated VNPs. As with genetic modifications, an enhancement in sensitivity was observed by the repetitive multimeric display of the biotin in the CP. A 15-fold increase was obtained. In this case we prove the possibility of detecting small molecules. Although these molecules may not all be therapeutical or detection relevant, the results indicate that the methods can successfully expose small molecules onto the VLPs surface and the approaches can easily be extended to load other small molecules like haptens. Methods for hapten detection are currently studied for their implication in allergies, inflammatory or autoimmune diseases (Omi, Ando et al. 2015). Therefore, a development in this direction seemed attractive. In the same path, chemically-conjugating peptides to the CP can also be important for the same purpose.

Although advances have been done in this direction through genetic modification of the VNPs, not all peptides are viable for generating chimeric VLPs, because of their size, isoelectric point or amino acid content. Hence, chemical conjugation could be the solution. A peptide that does not only interfered with the virus infection, but also hindered VLP assembly, was selected. The vasoactive intestinal peptide is a 28 amino acid neuropeptide implicated in inflammatory and autoimmune diseases (Venugopalan 1989). The possibility of obtaining functionalized nanoparticles with this peptide could be a major boost in the detection of autoimmune illnesses such as Chron's disease. Since the peptide does not have a cysteine, it was not possible to conjugate directly and create a disulfide bond. Therefore, a haloacetyl-NHS ester

heterobifunctional linker was used. This linker would allow the conjugation to the CP via a haloacetyl that would react to the cysteine's sulfhydryl group, resulting in a stable thioether linkage; and in the other end, a NHS ester that reacts with the peptide's N-terminus primary amine (Hermanson 2013). By SDS-PAGE and Western Blot we were able to confirm the peptide's conjugation to both TuMV VNPs. Future work will evolve around the use of these conjugated nanoparticles for diagnostics.

After seeing the possibilities of conjugation our VNP platform has, another potential application came into our attention. VLPs are an emerging class of targeted delivery vehicles with the potential to overcome the limitations of other nanoparticles (Yildiz, Lee et al. 2013, Glasgow and Tullman-Ercek 2014). In recent years, several groups have shown that VLPs can pack and deliver therapeutic cargo such as chemotherapeutic drugs, siRNA, RNA aptamers, proteins, and peptides (Ashley, Carnes et al. 2011, Lau, Baksh et al. 2011, Glasgow, Capehart et al. 2012, Anand, O'Neil et al. 2015, Kelly, Anand et al. 2015). Hence, we opted to conjugate an organoiridium complex. Iridium-based anticancer agents are currently attracting attention as potential anticancer agents with novel mechanisms of action (Gasser, Ott et al. 2011, Hearn, Romero-Canelón et al. 2013, Liu and Sadler 2014). The preliminary results confirmed the conjugation of the iridium (III) complex. These results lay the groundwork for further development of plant virus-based filamentous vehicles for use in cancer treatment. Previous studies reported that elongated viruses show enhanced tumor homing and tissue penetration than icosahedral VNPs (Geng, Dalhaimer et al. 2007, Shukla, Ablack et al. 2013, Chariou, Lee et al. 2016, Esfandiari, Arzanani et al. 2016), results consistent with those of rod-shaped synthetic nanoparticles (Chauhan, Popović et al. 2011). Elongated particles also present ligands in a more efficient manner. Cells are typically 10–100 times larger than a nanostructure, and the cell surface tends to be relatively flat. Therefore, an elongated structure could in theory interact with a larger number of binding sites on the cell surface, potentially increasing targeting

sensitivity and specificity. Nonetheless, there are still several variables that have to be taken into consideration like the stability, cell-entry and cargo deployment, as well as the cytotoxicity of these new VNPs.

In this thesis we displayed a diversity of applications of TuMV VNPs as scaffolds for multimeric display. Just with the information presented here, two different sites could be targeted, the N-terminus (by genetic modification) and the cysteine (by chemical conjugation). By using other bioconjugation chemistries, more residues (like lysines) could be exploited for this purpose. Further developments should appoint the combination of conjugation strategies in order to obtain multi-functional VNPs.

VNPs provide well-defined, stable and facile structures that can be readily converted into building blocks for the assembly of new nanoscale materials and devices. Structural features in the surface of the VNP are amenable to either chemical or genetic modifications to acquire novel functions. In addition, the possibility of producing VLPs that are non-infectious also constitute an advantage for several applications. Yet, the efforts outlined here represent only a fraction of the potential that viruses hold for nanotechnology. These studies have used the TuMV VNPs as scaffolds to display novel functions for enhanced applications in diagnostics or drug delivery. These are static attributes and do not convey any VNP action beyond the scaffolding function. Future advances will likely develop and expand upon dynamic biological traits to assemble multi-functional virus particles that respond to defined input signals derived from either their environment or a device interface. For example, generating VNPs that are able to sense, recognize and aim for a target, and then deliver a cargo, representing a new theranostic tool. This is just one of the countless possibilities that a multi-functional VNP can offer. This work lays the foundation for *Turnip mosaic virus*-derived nanoparticles to achieve this goal.

5. CONCLUSIONS

1. *Turnip mosaic virus* virions or VLPs can be developed in *Nicotiana benthamiana* plants.
2. These TuMV VNPs can be used as scaffolds for multimeric display of peptides or low-molecular-weight compounds.
3. There are at least two different conjugation sites: N-terminus (by genetic modification) and the cysteine residue (by chemical conjugation).
4. The multimeric, repetitive and organized display in VNPs enhance peptide and small compounds biological functions.
5. This platform can be used for detection of autoantibodies in serum.
6. Organometallic compounds, with antitumoral properties, can be conjugated to VNPs.
7. This study lays the groundwork for further conjugations and applications of modified *Turnip mosaic virus*-derived nanoparticles.

6. REFERENCES

Agirrezabala, X., E. Mendez-Lopez, G. Lasso, M. A. Sanchez-Pina, M. Aranda and M. Valle (2015). "The near-atomic cryoEM structure of a flexible filamentous plant virus shows homology of its coat protein with nucleoproteins of animal viruses." Elife **4**: e11795.

Agrios, G. N. (2005). chapter fourteen - PLANT DISEASES CAUSED BY VIRUSES. Plant Pathology (Fifth Edition). San Diego, Academic Press: 723-824.

Aller, M.-A., J.-L. Arias, I. Prieto, M. Losada and J. Arias (2010). "Bile duct ligation: step-by-step to cholangiocyte inflammatory tumorigenesis." European Journal of Gastroenterology & Hepatology **22**(6): 651-661.

Anand, P., A. O'Neil, E. Lin, T. Douglas and M. Holford (2015). "Tailored delivery of analgesic ziconotide across a blood brain barrier model using viral nanocontainers." Sci Rep **5**: 12497.

Ashley, C. E., E. C. Carnes, G. K. Phillips, P. N. Durfee, M. D. Buley, C. A. Lino, D. P. Padilla, B. Phillips, M. B. Carter, C. L. Willman, C. J. Brinker, J. d. C. Caldeira, B. Chackerian, W. Wharton and D. S. Peabody (2011). "Cell-Specific Delivery of Diverse Cargos by Bacteriophage MS2 Virus-Like Particles." ACS nano **5**(7): 5729-5745.

Ausar, S. F., T. R. Foubert, M. H. Hudson, T. S. Vedvick and C. R. Middaugh (2006). "Conformational stability and disassembly of Norwalk virus-like particles. Effect of pH and temperature." J Biol Chem **281**(28): 19478-19488.

Babin, C., N. Majeau and D. Leclerc (2013). "Engineering of papaya mosaic virus (PapMV) nanoparticles with a CTL epitope derived from influenza NP." Journal of Nanobiotechnology **11**: 10-10.

Baratova, L. A., A. V. Efimov, E. N. Dobrov, N. V. Fedorova, R. Hunt, G. A. Badun, A. L. Ksenofontov, L. Torrance and L. Jarvekulg (2001). "In situ spatial organization of Potato virus A coat protein subunits as assessed by tritium bombardment." J Virol **75**(20): 9696-9702.

Bárcena, J. and E. Blanco (2013). Design of Novel Vaccines Based on Virus-Like Particles or Chimeric Virions. Structure and Physics of Viruses: An Integrated Textbook. M. G. Mateu. Dordrecht, Springer Netherlands: 631-665.

Bataller, R. and D. A. Brenner (2005). "Liver fibrosis." The Journal of Clinical Investigation **115**(2): 209-218.

Bendahmane, M., M. Koo, E. Karrer and R. N. Beachy (1999). "Display of epitopes on the surface of tobacco mosaic virus: impact of charge and isoelectric point of the epitope on virus-host interactions." J Mol Biol **290**(1): 9-20.

Blechacz, B. R. A. and G. J. Gores "Cholangiocarcinoma." Clinics in Liver Disease **12**(1): 131-150.

Brunt, A. A. (1992). The general properties of potyviruses. Potyvirus Taxonomy. O. W. Barnett. Vienna, Springer Vienna: 3-16.

Burrell, C. J., P. Mackay, P. J. Greenaway, P. H. Hofschneider and K. Murray (1979). "Expression in *Escherichia coli* of hepatitis B virus DNA sequences cloned in plasmid pBR322." Nature **279**(5708): 43-47.

Caldeira, J. C. and D. S. Peabody (2011). "Thermal stability of RNA phage virus-like particles displaying foreign peptides." J Nanobiotechnology **9**: 22.

Carrington, J. C., D. D. Freed and T. C. Sanders (1989). "Autocatalytic processing of the potyvirus helper component proteinase in *Escherichia coli* and in vitro." J Virol **63**(10): 4459-4463.

Causey, L. D. and D. S. Dwyer (1996). "Detection of low affinity interactions between peptides and heat shock proteins by Chemiluminescence of Enhanced Avidity Reactions (CLEAR)." Nature Biotechnology **14**(3): 348-351.

Cerovska, N., H. Hoffmeisterova, T. Pecenkova, T. Moravec, H. Synkova, H. Plchova and J. Veleminsky (2008). "Transient expression of HPV16 E7 peptide (aa 44-60) and HPV16 L2 peptide (aa 108-120) on chimeric potyvirus-like particles using *Potato virus X*-based vector." Protein Expr Purif **58**(1): 154-161.

Comellas-Aragones, M., H. Engelkamp, V. I. Claessen, N. A. Sommerdijk, A. E. Rowan, P. C. Christianen, J. C. Maan, B. J. Verduin, J. J. Cornelissen and R. J. Nolte (2007). "A virus-based single-enzyme nanoreactor." Nat Nanotechnol **2**(10): 635-639.

Cornuz, J., S. Zwahlen, W. F. Jungi, J. Osterwalder, K. Klingler, G. van Melle, Y. Bangala, I. Guessous, P. Müller, J. Willers, P. Maurer, M. F. Bachmann and T. Cerny (2008). "A Vaccine against Nicotine for Smoking Cessation: A Randomized Controlled Trial." PLOS ONE **3**(6): e2547.

Cuenca, S., C. Mansilla, M. Aguado, C. Yuste-Calvo, F. Sánchez, J. M. Sánchez-Montero and F. Ponz (2016). "Nanonets derived from turnip mosaic virus as scaffolds for increased enzymatic activity of immobilized *Candida antarctica* lipase B." Frontiers in Plant Science **7**.

Chariou, P. L., K. L. Lee, J. K. Pokorski, G. M. Saidel and N. F. Steinmetz (2016). "Diffusion and Uptake of Tobacco Mosaic Virus as Therapeutic Carrier in Tumor Tissue: Effect of Nanoparticle Aspect Ratio." The Journal of Physical Chemistry B **120**(26): 6120-6129.

Charudattan, R. and E. Hiebert (2007). "A Plant Virus as a Bioherbicide for Tropical Soda Apple, *Solanum Viarum*." Outlooks on Pest Management **18**(4): 167-171.

Chatterji, A., L. L. Burns, S. S. Taylor, G. P. Lomonosoff, J. E. Johnson, T. Lin and C. Porta (2003). "Cowpea Mosaic Virus: From the Presentation of Antigenic Peptides to the Display of Active Biomaterials." Intervirology **45**(4-6): 362-370.

Chauhan, V. P., Z. Popović, O. Chen, J. Cui, D. Fukumura, M. G. Bawendi and R. K. Jain (2011). "Fluorescent Nanorods and Nanospheres for Real-Time In Vivo Probing of Nanoparticle Shape-Dependent Tumor Penetration." Angewandte Chemie (International Ed. in English) **50**(48): 11417-11420.

Chen, Q. and H. Lai (2013). "Plant-derived virus-like particles as vaccines." Hum Vaccin Immunother **9**(1): 26-49.

Chung, C. and Y. Iwakiri (2013). "The lymphatic vascular system in liver diseases: its role in ascites formation." Clin Mol Hepatol **19**(2): 99-104.

D'Aoust, M. A., M. M. Couture, N. Charland, S. Trepanier, N. Landry, F. Ors and L. P. Vezina (2010). "The production of hemagglutinin-based virus-like particles in plants: a rapid, efficient and safe response to pandemic influenza." Plant Biotechnol J **8**(5): 607-619.

D'Aoust, M. A., P. O. Lavoie, M. M. Couture, S. Trepanier, J. M. Guay, M. Dargis, S. Mongrand, N. Landry, B. J. Ward and L. P. Vezina (2008). "Influenza virus-like particles produced by transient expression in *Nicotiana benthamiana* induce a protective immune response against a lethal viral challenge in mice." Plant Biotechnol J **6**(9): 930-940.

Dedeo, M. T., D. T. Finley and M. B. Francis (2011). "Viral capsids as self-assembling templates for new materials." Prog Mol Biol Transl Sci **103**: 353-392.

Delchambre, M., D. Gheysen, D. Thines, C. Thiriart, E. Jacobs, E. Verdin, M. Horth, A. Burny and F. Bex (1989). "The GAG precursor of simian immunodeficiency virus assembles into virus-like particles." The EMBO Journal **8**(9): 2653-2660.

Dickmeis, C., M. M. Honickel, R. Fischer and U. Commandeur (2015). "Production of Hybrid Chimeric PVX Particles Using a Combination of TMV and PVX-Based Expression Vectors." Front Bioeng Biotechnol **3**: 189.

Duval, F., D. E. Cruz-Vega, I. González-Gamboa, M. T. González-Garza, F. Ponz, F. Sánchez, G. Alarcón-Galván and J. E. Moreno-Cuevas (2016). "Detection of autoantibodies to vascular endothelial growth factor receptor-3 in bile duct ligated rats and correlations with a panel of traditional markers of liver diseases." Disease Markers.

Duval, F., D. E. Cruz-Vega, I. González-Gamboa, M. T. González-Garza, F. Ponz, F. Sánchez, G. Alarcón-Galván and J. E. Moreno-Cuevas (2016). "Detection of autoantibodies to vascular endothelial growth factor receptor-3 in bile duct ligated rats and correlations with a panel of traditional markers of liver diseases." Disease Markers **2016**: 7.

Duval, F., J. E. Moreno-Cuevas, M. T. González-Garza, C. Maldonado-Bernal and D. E. Cruz-Vega (2015). "Liver Fibrosis and Mechanisms of the Protective Action of Medicinal Plants Targeting Inflammation and the Immune Response." International Journal of Inflammation **2015**: 943497.

Esfandiari, N., M. K. Arzanani, M. Soleimani, M. Kohi-Habibi and W. E. Svendsen (2016). "A new application of plant virus nanoparticles as drug delivery in breast cancer." Tumour Biol **37**(1): 1229-1236.

Fauquet, C. M. and D. Fargette (2005). "International Committee on Taxonomy of Viruses and the 3,142 unassigned species." Virology Journal **2**: 64-64.

Fauquet, C. M. and G. P. Martelli (2001). *Viral Classification and Nomenclature*. eLS, John Wiley & Sons, Ltd.

Fava, G., S. Demorrow, E. Gaudio, A. Franchitto, P. Onori, G. Carpino, S. Glaser, H. Francis, M. Coufal, L. Marucci, D. Alvaro, M. Marzioni, T. Horst, R. Mancinelli, A. Benedetti and G. Alpini (2009). "Endothelin inhibits cholangiocarcinoma growth by a decrease in the vascular endothelial growth factor expression." Liver Int **29**(7): 1031-1042.

Fernández-Fernández, M. R., J. L. Martínez-Torrecuadrada, J. I. Casal and J. A. García (1998). "Development of an antigen presentation system based on plum pox potyvirus." FEBS Lett **427**(2): 229-235.

Fernández-Fernández, M. R., J. L. Martínez-Torrecuadrada, F. Roncal, E. Domínguez and J. A. García (2002). "Identification of immunogenic hot spots within plum pox potyvirus capsid protein for efficient antigen presentation." JOURNAL OF VIROLOGY **76**(24): 12646-12653.

Fodje, M. N. and S. Al-Karadaghi (2002). "Occurrence, conformational features and amino acid propensities for the π -helix." Protein Engineering, Design and Selection **15**(5): 353-358.

Franchitto, A., P. Onori, A. Renzi, G. Carpino, R. Mancinelli, D. Alvaro and E. Gaudio (2013). "Expression of vascular endothelial growth factors and their receptors by hepatic progenitor cells in human liver diseases." Hepatobiliary Surg Nutr **2**(2): 68-77.

Gasser, G., I. Ott and N. Metzler-Nolte (2011). "Organometallic anticancer compounds." J Med Chem **54**(1): 3-25.

Gaudio, E., B. Barbaro, D. Alvaro, S. Glaser, H. Francis, Y. Ueno, C. J. Meininger, A. Franchitto, P. Onori, M. Marzioni, S. Taffetani, G. Fava, G. Stoica, J. Venter, R. Reichenbach, S. De Morrow, R. Summers and G. Alpini (2006). "Vascular endothelial growth factor stimulates rat cholangiocyte proliferation via an autocrine mechanism." Gastroenterology **130**(4): 1270-1282.

Geiger, F. C., F. J. Eber, S. Eiben, A. Mueller, H. Jeske, J. P. Spatz and C. Wege (2013). "TMV nanorods with programmed longitudinal domains of differently addressable coat proteins." Nanoscale **5**(9): 3808-3816.

Geng, Y., P. Dalhaimer, S. Cai, R. Tsai, M. Tewari, T. Minko and D. E. Discher (2007). "Shape effects of filaments versus spherical particles in flow and drug delivery." Nat Nanotechnol **2**(4): 249-255.

Gerald, S., K. Amy, M. Michele, B. Wen, B. Timothy, B. Sarah, M. Ian, S. Jian, S. Phoebe, B. Esther, G. David and G. Said (2008). Flexible filamentous virus structures from fiber diffraction, ICDD.

Giddings, G. (2001). "Transgenic plants as protein factories." CURRENT OPINION IN BIOTECHNOLOGY **12**(5): 450-454.

Glasgow, J. and D. Tullman-Ercek (2014). "Production and applications of engineered viral capsids." Appl Microbiol Biotechnol **98**(13): 5847-5858.

Glasgow, J. E., S. L. Capehart, M. B. Francis and D. Tullman-Ercek (2012). "Osmolyte-Mediated Encapsulation of Proteins inside MS2 Viral Capsids." ACS Nano **6**(10): 8658-8664.

Gonzalez, I. (2011). An epitope-presentation system: A nanotechnological approach to a biological paradigm. Master in Agroforestry Biotechnology Master, UPM.

Gowda, S., P. B. Desai, V. V. Hull, A. A. K. Math, S. N. Vernekar and S. S. Kulkarni (2009). "A review on laboratory liver function tests." The Pan African medical journal **3**: 17.

Grgacic, E. V. and D. A. Anderson (2006). "Virus-like particles: passport to immune recognition." Methods **40**(1): 60-65.

Grill, L. K., K. E. Palmer and G. P. Pogue (2005). "Use of plant viruses for production of plant-derived vaccines." Critical Reviews in Plant Sciences **24**(4): 309-323.

Gutiérrez, P., S. Bastos-Aristizábal and M. Marín (2011). "MODELACIÓN ESTRUCTURAL DE LA PROTEÍNA DE LA CÁPSIDE DEL VIRUS A DE LA PAPA (PVA, POTYVIRUS)." Actualidades Biológicas **33**: 93-102.

Hansen, S. and C. M. Lehr (2012). "Nanoparticles for transcutaneous vaccination." Microb Biotechnol **5**(2): 156-167.

Hearn, J. M., I. Romero-Canelón, B. Qamar, Z. Liu, I. Hands-Portman and P. J. Sadler (2013).

"Organometallic Iridium(III) Anticancer Complexes with New Mechanisms of Action: NCI-60 Screening, Mitochondrial Targeting, and Apoptosis." ACS Chemical Biology **8**(6): 1335-1343.

Helzberg, J. H., J. M. Petersen and J. L. Boyer (1987). "Improved survival with primary sclerosing cholangitis. A review of clinicopathologic features and comparison of symptomatic and asymptomatic patients." Gastroenterology **92**(6): 1869-1875.

Henkel, M., N. Röckendorf and A. Frey (2016). "Selective and Efficient Cysteine Conjugation by Maleimides in the Presence of Phosphine Reductants." Bioconjugate Chemistry **27**(10): 2260-2265.

Hermanson, G. T. (2013). Chapter 1 - Introduction to Bioconjugation. Bioconjugate Techniques (Third edition). Boston, Academic Press: 1-125.

Hermanson, G. T. (2013). Chapter 6 - Heterobifunctional Crosslinkers. Bioconjugate Techniques (Third edition). Boston, Academic Press: 299-339.

Himoto, T., S. Kuriyama, J. Y. Zhang, E. K. Chan, Y. Kimura, T. Masaki, N. Uchida, M. Nishioka and E. M. Tan (2005). "Analyses of autoantibodies against tumor-associated antigens in patients with hepatocellular carcinoma." Int J Oncol **27**(4): 1079-1085.

Himoto, T. and M. Nishioka (2013). "Autoantibodies in liver disease: important clues for the diagnosis, disease activity and prognosis." Auto Immun Highlights **4**(2): 39-53.

Hong, Y. and J. Huang (2015). "Autoantibodies against tumor-associated antigens for detection of hepatocellular carcinoma." World Journal of Hepatology **7**(11): 1581-1585.

Huang, Z., G. Elkin, B. J. Maloney, N. Beuhner, C. J. Arntzen, Y. Thanavala and H. S. Mason (2005). "Virus-like particle expression and assembly in plants: hepatitis B and Norwalk viruses." Vaccine **23**(15): 1851-1858.

Ibort, P. (2011). Turnip mosaic virus-based nanoplatfoms for applications in nanobiotechnology. Master in Agroforestry Biotechnology Master, UPM.

Iizuka, A., M. Komiyama, C. Oshita, A. Kume, T. Ashizawa, K. Mitsuya, N. Hayashi, Y. Nakasu, K. Yamaguchi and Y. Akiyama (2014). "Anti-vascular endothelial growth factor receptor (VEGFR) 2 autoantibody identification in glioblastoma patient using single B cell-based antibody gene cloning." Immunol Lett **159**(1-2): 15-22.

Iredale, J. P., A. Thompson and N. C. Henderson (2013). "Extracellular matrix degradation in liver fibrosis: Biochemistry and regulation." Biochim Biophys Acta **1832**(7): 876-883.

Jagadish, M., C. W. Ward, K. H. Gough, P. A. Tulloch, L. A. Whittaker and D. D. Shukla (1991). "Expression of potyvirus coat protein in *Escherichia coli* and yeast and its assembly into virus-like particles." Journal of General Virology **72**: 1543-1550.

Jennings, G. T. and M. F. Bachmann (2008). "The coming of age of virus-like particle vaccines." Biol Chem **389**(5): 521-536.

Jia, J. B., P. Y. Zhuang, H. C. Sun, J. B. Zhang, W. Zhang, X. D. Zhu, Y. Q. Xiong, H. X. Xu and Z. Y. Tang (2009). "Protein expression profiling of vascular endothelial growth factor and its receptors identifies subclasses of hepatocellular carcinoma and predicts survival." J Cancer Res Clin Oncol **135**(6): 847-854.

Jiang, L., Q. Li, M. Li, Z. Zhou, L. Wu, J. Fan, Q. Zhang, H. Zhu and Z. Xu (2006). "A modified TMV-based vector facilitates the expression of longer foreign epitopes in tobacco." Vaccine **24**(2): 109-115.

Johnson, J., T. Lin and G. Lomonossoff (1997). "Presentation of heterologous peptides on plant viruses." Annual Review of Phytopathology **35**: 67-86.

Jordan, R. (1992). Potyviruses, monoclonal antibodies, and antigenic sites. Potyvirus Taxonomy. O. W. Barnett. Vienna, Springer Vienna: 81-95.

Jordan, R. and J. Hammond (1991). "Comparison and differentiation of potyvirus isolates and identification of strain-, virus-, subgroup-specific and potyvirus group-common epitopes using monoclonal antibodies." Journal of General Virology **72**(1): 25-36.

Jüngst, C., T. Berg, J. Cheng, R. M. Green, J. Jia, A. L. Mason and F. Lammert (2013). "Intrahepatic cholestasis in common chronic liver diseases." European Journal of Clinical Investigation **43**(10): 1069-1083.

Kalinciema, I., D. Skrastina, V. Ose, P. Pumpens and A. Zeltins (2012). "Potato virus Y-like particles as a new carrier for the presentation of foreign protein stretches." Mol Biotechnol **52**(2): 129-139.

Kaltgrad, E., M. K. O'Reilly, L. Liao, S. Han, J. C. Paulson and M. G. Finn (2008). "On-Virus Construction of Polyvalent Glycan Ligands for Cell-Surface Receptors." Journal of the American Chemical Society **130**(14): 4578-4579.

Kang, K. and J.-S. Lim (2012). "Induction of Functional Changes of Dendritic Cells by Silica Nanoparticles." Immune Network **12**(3): 104-112.

Kelly, P., P. Anand, A. Uvaydov, S. Chakravartula, C. Sherpa, E. Pires, A. O'Neil, T. Douglas and M. Holford (2015). "Developing a Dissociative Nanocontainer for Peptide Drug Delivery." Int J Environ Res Public Health **12**(10): 12543-12555.

Kendall, A., M. McDonald, W. Bian, T. Bowles, S. C. Baumgarten, J. Shi, P. L. Stewart, E. Bullitt, D. Gore, T. C. Irving, W. M. Havens, S. A. Ghabrial, J. S. Wall and G. Stubbs (2008). "Structure of flexible filamentous plant viruses." J Virol **82**(19): 9546-9554.

Kim, J., J. E. Lee, S. H. Lee, J. H. Yu, J. H. Lee, T. G. Park and T. Hyeon (2008). "Designed Fabrication of a Multifunctional Polymer Nanomedical Platform for Simultaneous Cancer- Targeted Imaging and Magnetically Guided Drug Delivery." Advanced Materials **20**(3): 478-483.

Koch, C., K. Wabbel, F. J. Eber, P. Krolla-Sidenstein, C. Azucena, H. Gliemann, S. Eiben, F. Geiger and C. Wege (2015). "Modified TMV Particles as Beneficial Scaffolds to Present Sensor Enzymes." Front Plant Sci **6**: 1137.

Kong, Q., L. Richter, Y. F. Yang, C. J. Arntzen, H. S. Mason and Y. Thanavala (2001). "Oral immunization with hepatitis B surface antigen expressed in transgenic plants." Proceedings of the National Academy of Sciences of the United States of America **98**(20): 11539-11544.

Kumar, S., W. Ochoa, P. Singh, C. Hsu, A. Schneemann, M. Manchester, M. Olson and V. Reddy (2009). "Tomato bushy stunt virus (TBSV), a versatile platform for polyvalent display of antigenic epitopes and vaccine design." Virology **388**(1): 185-190.

Kushnir, N., S. J. Streatfield and V. Yusibov (2012). "Virus-like particles as a highly efficient vaccine platform: diversity of targets and production systems and advances in clinical development." Vaccine **31**(1): 58-83.

Landesberg, R., A. Burke, D. Pinsky, R. Katz, J. Vo, S. B. Eisig and H. H. Lu (2005). "Activation of platelet-rich plasma using thrombin receptor agonist peptide." J Oral Maxillofac Surg **63**(4): 529-535.

Lau, J. L., M. M. Baksh, J. D. Fiedler, S. D. Brown, A. Kussrow, D. J. Bornhop, P. Ordoukhanian and M. G. Finn (2011). "Evolution and Protein Packaging of Small-Molecule RNA Aptamers." ACS Nano **5**(10): 7722-7729.

Le, D. H., K. L. Lee, S. Shukla, U. Commandeur and N. F. Steinmetz (2017). "Potato virus X, a filamentous plant viral nanoparticle for doxorubicin delivery in cancer therapy." Nanoscale **9**(6): 2348-2357.

Lee, K. L., K. Uhde-Holzem, R. Fischer, U. Commandeur and N. F. Steinmetz (2014). "Genetic engineering and chemical conjugation of potato virus X." Methods Mol Biol **1108**: 3-21.

Lewis, J. D., G. Destito, A. Zijlstra, M. J. Gonzalez, J. P. Quigley, M. Manchester and H. Stuhlmann (2006). "Viral nanoparticles as tools for intravital vascular imaging." Nature medicine **12**(3): 354-360.

Leyva-Illades, D., M. McMillin, M. Quinn and S. Demorrow (2012). "Cholangiocarcinoma pathogenesis: Role of the tumor microenvironment." Transl Gastrointest Cancer **1**(1): 71-80.

Li, L., J. Wu and C. Gao (2011). "Gradient immobilization of a cell adhesion RGD peptide on thermal responsive surface for regulating cell adhesion and detachment." Colloids Surf B Biointerfaces **85**(1): 12-18.

Lico, C., E. Benvenuto and S. Baschieri (2015). "The Two-Faced Potato Virus X: From Plant Pathogen to Smart Nanoparticle." Front Plant Sci **6**: 1009.

Lico, C., F. Capuano, G. Renzone, M. Donini, C. Marusic, A. Scaloni, E. Benvenuto and S. Baschieri (2006). "Peptide display on Potato virus X: molecular features of the coat protein-fused peptide affecting cell-to-cell and phloem movement of chimeric virus particles." Journal of General Virology **87**: 3103-3112.

Limpaiboon, T., B. Sripa, S. Wongkham, V. Bhudhisawasdi, S. Chau-in and Y. Teerajetgul (2004). "Anti-p53 antibodies and p53 protein expression in cholangiocarcinoma." Hepatogastroenterology **51**(55): 25-28.

Liu, T., L. Li, X. Teng, X. Huang, H. Liu, D. Chen, J. Ren, J. He and F. Tang (2011). "Single and repeated dose toxicity of mesoporous hollow silica nanoparticles in intravenously exposed mice." Biomaterials **32**(6): 1657-1668.

Liu, Z., A. Habtemariam, A. M. Pizarro, G. J. Clarkson and P. J. Sadler (2011). "Organometallic Iridium(III) Cyclopentadienyl Anticancer Complexes Containing C,N-Chelating Ligands." Organometallics **30**(17): 4702-4710.

Liu, Z. and P. J. Sadler (2014). "Organoiridium complexes: anticancer agents and catalysts." Acc Chem Res **47**(4): 1174-1185.

Lleo, A. (2014). Chapter 2 - What Is an Autoantibody? A2 - Shoenfeld, Yehuda. Autoantibodies (Third Edition). P. L. Meroni and M. E. Gershwin. San Diego, Elsevier: 13-20.

Maroni, L., B. Haibo, D. Ray, T. Zhou, Y. Wan, F. Meng, M. Marzioni and G. Alpini (2015). "Functional and Structural Features of Cholangiocytes in Health and Disease." Cellular and Molecular Gastroenterology and Hepatology **1**(4): 368-380.

Martínez-Herrera, D., J. Romero, J. M. Martínez-Zapater and F. Ponz (1994). "Suitability of *Arabidopsis thaliana* as a system for the study of plant-virus interactions." Fitopatología **29**: 132-136.

Martorell, L., J. Martínez-González, C. Rodríguez, M. Gentile, O. Calvayrac and L. Badimon (2008). "Thrombin and protease-activated receptors (PARs) in atherothrombosis." Thrombosis and Haemostasis **99**(2): 305-315.

Mason, H. S., J. M. Ball, J. J. Shi, X. Jiang, M. K. Estes and C. J. Arntzen (1996). "Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice." Proceedings of the National Academy of Sciences of the United States of America **93**(11): 5335-5340.

Mateu, M. G. (2011). "Virus engineering: functionalization and stabilization." Protein Engineering, Design and Selection **24**(1-2): 53-63.

McCormick, A. A. and K. E. Palmer (2008). "Genetically engineered Tobacco mosaic virus as nanoparticle vaccines." Expert Rev Vaccines **7**(1): 33-41.

McDonald, M., A. Kendall, W. Bian, I. McCullough, E. Lio, W. M. Havens, S. A. Ghabrial and G. Stubbs (2010). "Architecture of the potyviruses." Virology **405**(2): 309-313.

Meier, W. (1999). "Nanostructure synthesis using surfactants and copolymers." Current Opinion in Colloid & Interface Science **4**(1): 6-14.

Mesa, V. A., R. De Vos and J. Fevery (1997). "Elevation of the serum bilirubin diconjugate fraction provides an early marker for cholestasis in the rat." J Hepatol **27**(5): 912-916.

Mody, V. V., A. Cox, S. Shah, A. Singh, W. Bevins and H. Parihar (2014). "Magnetic nanoparticle drug delivery systems for targeting tumor." Applied Nanoscience **4**(4): 385-392.

Mori, M., H. Fujii, T. Ogawa, S. Kobayashi, S. Iwai, H. Morikawa, M. Enomoto, A. Tamori, A. Sawada, S. Takeda and N. Kawada (2011). "Close correlation of liver stiffness with collagen deposition and presence of myofibroblasts in non-alcoholic fatty liver disease." Hepatol Res **41**(9): 897-903.

Murawski, M. R., L. W. McGinnes, R. W. Finberg, E. A. Kurt-Jones, M. J. Massare, G. Smith, P. M. Heaton, A. E. Fraire and T. G. Morrison (2010). "Newcastle disease virus-like particles containing respiratory syncytial virus G protein induced protection in BALB/c mice, with no evidence of immunopathology." J Virol **84**(2): 1110-1123.

Mustafa, M. Z., V. H. Nguyen, F. Le Naour, E. De Martin, E. Beleoken, C. Guettier, C. Johanet, D. Samuel, J.-C. Duclos-Vallee and E. Ballot (2016). "Autoantibody signatures defined by serological proteome analysis in sera from patients with cholangiocarcinoma." Journal of Translational Medicine **14**: 17.

Nagele, E. P., M. Han, N. K. Acharya, C. DeMarshall, M. C. Kosciuk and R. G. Nagele (2013). "Natural IgG autoantibodies are abundant and ubiquitous in human sera, and their number is influenced by age, gender, and disease." PLoS One **8**(4): e60726.

Newman, M., F. M. Suk, M. Cajimat, P. K. Chua and C. Shih (2003). "Stability and morphology comparisons of self-assembled virus-like particles from wild-type and mutant human hepatitis B virus capsid proteins." Journal of Virology **77**(24): 12950-12960.

Nolasco, G., C. De Blas, V. Torres and F. Ponz (1993). "A method combining immunocapture and PCR amplification in a microtiter plate for the detection of plant viruses and subviral pathogens." Journal of Virological Methods **45**(2): 201-218.

Omi, K., T. Ando, T. Sakyu, T. Shirakawa, Y. Uchida, A. Oka, N. Ise, K. Aoyagi and K. Goishi (2015). "Noncompetitive immunoassay detection system for haptens on the basis of antimitotype antibodies." Clin Chem **61**(4): 627-635.

Peabody, D. S., B. Manifold-Wheeler, A. Medford, S. K. Jordan, J. d. C. Caldeira and B. Chackerian (2008). "Immunogenic display of diverse peptides on virus-like particles of RNA phage MS2." Journal of molecular biology **380**(1): 252-263.

Penz-Österreicher, M., C. H. Österreicher and M. Trauner (2011). "Fibrosis in Autoimmune and Cholestatic Liver Disease." Best Practice & Research. Clinical Gastroenterology **25**(2-4): 245-258.

Peyret, H. and G. Lomonossoff (2013). "The pEAQ vector series: the easy and quick way to produce recombinant proteins in plants." Plant Molecular Biology **83**(1-2): 51-58.

Pokorski, J. K. and N. F. Steinmetz (2011). "The art of engineering viral nanoparticles." Mol Pharm **8**(1): 29-43.

Poole, L. B. (2015). "The basics of thiols and cysteines in redox biology and chemistry." Free Radic Biol Med **80**: 148-157.

Porta, C., V. E. Spall, K. C. Findlay, R. C. Gergerich, C. E. Farrance and G. P. Lomonossoff (2003). "Cowpea mosaic virus-based chimaeras - Effects of inserted peptides on the phenotype, host range, and transmissibility of the modified viruses." VIROLOGY **310**(1): 50-63.

Pushko, P., P. Pumpens and E. Grens (2013). "Development of virus-like particle technology from small highly symmetric to large complex virus-like particle structures." Intervirology **56**(3): 141-165.

Qin, L. X. and Z. Y. Tang (2003). "Hepatocellular carcinoma with obstructive jaundice: diagnosis, treatment and prognosis." World J Gastroenterol **9**(3): 385-391.

Ramqvist, T., K. Andreasson and T. Dalianis (2007). "Vaccination, immune and gene therapy based on virus-like particles against viral infections and cancer." Expert Opinion on Biological Therapy **7**(7): 997-1007.

Reynaert, H., M. G. Thompson, T. Thomas and A. Geerts (2002). "Hepatic stellate cells: role in microcirculation and pathophysiology of portal hypertension." Gut **50**(4): 571-581.

Riechmann, J. L., S. Lain and J. A. Garcia (1992). "Highlights and prospects of potyvirus molecular biology." J Gen Virol **73** (Pt 1): 1-16.

Rockey, D. C., S. H. Caldwell, Z. D. Goodman, R. C. Nelson and A. D. Smith (2009). "Liver biopsy." Hepatology **49**(3): 1017-1044.

Roco, M. C. (2003). "Converging science and technology at the nanoscale: opportunities for education and training." Nat Biotech **21**(10): 1247-1249.

Rohovie, M. J., M. Nagasawa and J. R. Swartz (2017). "Virus-like particles: Next-generation nanoparticles for targeted therapeutic delivery." Bioengineering & Translational Medicine **2**(1): 43-57.

Roy, P. and R. Noad (2009). "Virus-like particles as a vaccine delivery system: myths and facts." Adv Exp Med Biol **655**: 145-158.

Royston, E., A. Ghosh, P. Kofinas, M. T. Harris and J. N. Culver (2008). "Self-Assembly of Virus-Structured High Surface Area Nanomaterials and Their Application as Battery Electrodes." Langmuir **24**(3): 906-912.

Rucksaken, R., C. Pairojkul, P. Pinlaor, N. Khuntikeo, S. Roytrakul, C. Selmi and S. Pinlaor (2014). "Plasma Autoantibodies against Heat Shock Protein 70, Enolase 1 and Ribonuclease/Angiogenin Inhibitor 1 as Potential Biomarkers for Cholangiocarcinoma." PLOS ONE **9**(7): e103259.

Sainsbury, F., M. C. Canizares and G. P. Lomonossoff (2010). "Cowpea mosaic virus: the plant virus-based biotechnology workhorse." Annu Rev Phytopathol **48**: 437-455.

Sainsbury, F. and G. P. Lomonossoff (2008). "Extremely high-level and rapid transient protein production in plants without the use of viral replication." Plant Physiol **148**(3): 1212-1218.

Sainsbury, F., E. C. Thuenemann and G. P. Lomonossoff (2009). "pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants." Plant Biotechnol J **7**(7): 682-693.

Sanchez-Sanchez, L., R. D. Cadena-Nava, L. A. Palomares, J. Ruiz-Garcia, M. S. Koay, J. J. Cornelissen and R. Vazquez-Duhalt (2014). "Chemotherapy pro-drug activation by biocatalytic virus-like nanoparticles containing cytochrome P450." Enzyme Microb Technol **60**: 24-31.

Sánchez, F., D. Martínez-Herrera, I. Aguilar and F. Ponz (1998). "Infectivity of turnip mosaic potyvirus cDNA clones and transcripts on the systemic host *Arabidopsis thaliana* and local lesion hosts." Virus Res **55**(2): 207-219.

Sanchez, F., M. Saez, P. Lunello and F. Ponz (2013). "Plant viral elongated nanoparticles modified for log-increases of foreign peptide immunogenicity and specific antibody detection." J Biotechnol **168**(4): 409-415.

Sánchez, F., X. Wang, C. E. Jenner, J. A. Walsh and F. Ponz (2003). "Strains of *Turnip mosaic potyvirus* as defined by the molecular analysis of the coat protein gene of the virus." VIRUS RESEARCH **94**(1): 33-43.

Santi, L., L. Batchelor, Z. Huang, B. Hjelm, J. Kilbourne, C. J. Arntzen, Q. Chen and H. S. Mason (2008). "An efficient plant viral expression system generating orally immunogenic Norwalk virus-like particles." Vaccine **26**(15): 1846-1854.

Santi, L., Z. Huang and H. Mason (2006). "Virus-like particles production in green plants." Methods **40**(1): 66-76.

Schielke, A., M. Filter, B. Appel and R. Johne (2011). "Thermal stability of hepatitis E virus assessed by a molecular biological approach." Virol J **8**: 487.

Shapiro, J. M., H. Smith and F. Schaffner (1979). "Serum bilirubin: a prognostic factor in primary biliary cirrhosis." Gut **20**(2): 137-140.

Shifeng, H., W. Danni, C. Pu, Y. Ping, C. Ju and Z. Liping (2013). "Circulating Liver-Specific miR-122 as a Novel Potential Biomarker for Diagnosis of Cholestatic Liver Injury." PLOS ONE **8**(9): e73133.

Shukla, D. D., C. W. Ward and A. A. Brunt (1994). The Potyviridae. Wallingford, CAB INTERNATIONAL.
Shukla, S., A. L. Ablack, A. M. Wen, K. L. Lee, J. D. Lewis and N. F. Steinmetz (2013). "Increased Tumor Homing and Tissue Penetration of the Filamentous Plant Viral Nanoparticle Potato virus X." Molecular Pharmaceutics **10**(1): 33-42.

Smith, M. L., J. A. Lindbo, S. Dillard-Telm, P. M. Brosio, A. B. Lasnik, A. A. McCormick, L. V. Nguyen and K. E. Palmer (2006). "Modified *Tobacco mosaic virus* particles as scaffolds for display of protein antigens for vaccine applications." Virology **348**(2): 475-488.

Soto, C. M., A. S. Blum, G. J. Vora, N. Lebedev, C. E. Meador, A. P. Won, A. Chatterji, J. E. Johnson and B. R. Ratna (2006). "Fluorescent Signal Amplification of Carbocyanine Dyes Using Engineered Viral Nanoparticles." Journal of the American Chemical Society **128**(15): 5184-5189.

Spence, N. J. (1992). The identification, distribution and ecology of bean common mosaic virus in Africa. PhD Thesis, University of Birmingham, UK.

Steele, J. F. C., H. Peyret, K. Saunders, R. Castells-Graells, J. Marsian, Y. Meshcheriakova and G. P. Lomonosoff (2017). "Synthetic plant virology for nanobiotechnology and nanomedicine." Wiley Interdiscip Rev Nanomed Nanobiotechnol.

Steinmetz, N. F., M. E. Mertens, R. E. Taurog, J. E. Johnson, U. Commandeur, R. Fischer and M. Manchester (2010). "Potato virus X as a novel platform for potential biomedical applications." Nano Lett **10**(1): 305-312.

Strable, E. and M. G. Finn (2009). Chemical Modification of Viruses and Virus-Like Particles. Viruses and Nanotechnology. M. Manchester and N. F. Steinmetz. Berlin, Heidelberg, Springer Berlin Heidelberg: 1-21.

Stubbs, G., A. Kendall, M. McDonald, W. Bian, T. Bowles, S. Baumgarten, I. McCullough, J. Shi, P. Stewart, E. Bullitt, D. Gore and S. Ghabrial (2012). "Flexible filamentous virus structures from fiber diffraction." Powder Diffraction **23**(02): 113-117.

Tang, F., L. Li and D. Chen (2012). "Mesoporous Silica Nanoparticles: Synthesis, Biocompatibility and Drug Delivery." Advanced Materials **24**(12): 1504-1534.

Tissot, A. C., R. Renhofa, N. Schmitz, I. Cielens, E. Meijerink, V. Ose, G. T. Jennings, P. Saudan, P. Pumpens and M. F. Bachmann (2010). "Versatile virus-like particle carrier for epitope based vaccines." PLoS ONE **5**(3): e9809.

Touriño, A., F. Sánchez, A. Fereres and F. Ponz (2008). "High expression of foreign proteins from a biosafe viral vector derived from *Turnip mosaic virus*." Spanish Journal of Agricultural Research **6**: 48-58.

Tugues, S., M. Morales-Ruiz, G. Fernandez-Varo, J. Ros, D. Arteta, J. Munoz-Luque, V. Arroyo, J. Rodes and W. Jimenez (2005). "Microarray analysis of endothelial differentially expressed genes in liver of cirrhotic rats." Gastroenterology **129**(5): 1686-1695.

Urcuqui-Inchima, S., A. L. Haenni and F. Bernardi (2001). "Potyvirus proteins: a wealth of functions." Virus Research **74**(1-2): 157-175.

Vallhov, H., S. Gabrielsson, M. Strømme, A. Scheynius and A. E. Garcia-Bennett (2007). "Mesoporous Silica Particles Induce Size Dependent Effects on Human Dendritic Cells." Nano Letters **7**(12): 3576-3582.

Veidal, S. S., E. Vassiliadis, N. Barascuk, C. Zhang, T. Segovia-Silvestre, L. Klickstein, M. R. Larsen, P. Qvist, C. Christiansen, B. Vainer and M. A. Karsdal (2010). "Matrix metalloproteinase-9-mediated type III collagen degradation as a novel serological biochemical marker for liver fibrogenesis." Liver Int **30**(9): 1293-1304.

Venugopalan, C. S. (1989). "Vasoactive intestinal peptide (VIP), a putative neurotransmitter of nonadrenergic, noncholinergic (NANC) inhibitory innervation and its relevance to therapy." Journal of Veterinary Pharmacology and Therapeutics **12**(2): 113-123.

Verchot, J., E. V. Koonin and J. C. Carrington (1991). "The 35-kDa protein from the N-terminus of the potyviral polyprotein functions as a third virus-encoded proteinase." Virology **185**(2): 527-535.

Wang, H. and G. Stubbs (1994). "Structure Determination of Cucumber Green Mottle Mosaic Virus by X-ray Fiber Diffraction." Journal of Molecular Biology **239**(3): 371-384.

Wang, J. J., B. J. S. Sanderson and H. Wang (2007). "Cytotoxicity and genotoxicity of ultrafine crystalline SiO₂ particulate in cultured human lymphoblastoid cells." Environmental and Molecular Mutagenesis **48**(2): 151-157.

Wang, Q., T. R. Chan, R. Hilgraf, V. V. Fokin, K. B. Sharpless and M. G. Finn (2003). "Bioconjugation by Copper(I)-Catalyzed Azide-Alkyne [3 + 2] Cycloaddition." Journal of the American Chemical Society **125**(11): 3192-3193.

Wen, A. M., P. H. Rambhia, R. H. French and N. F. Steinmetz (2013). "Design rules for nanomedical engineering: from physical virology to the applications of virus-based materials in medicine." J Biol Phys **39**(2): 301-325.

Yamauchi, Y., K. Michitaka and M. Onji (1998). "Morphometric analysis of lymphatic and blood vessels in human chronic viral liver diseases." Am J Pathol **153**(4): 1131-1137.

Yang, S., T. Wang, J. Bohon, M. E. Gagne, M. Bolduc, D. Leclerc and H. Li (2012). "Crystal structure of the coat protein of the flexible filamentous papaya mosaic virus." J Mol Biol **422**(2): 263-273.

Yi, H., S. Nisar, S.-Y. Lee, M. A. Powers, W. E. Bentley, G. F. Payne, R. Ghodssi, G. W. Rubloff, M. T. Harris and J. N. Culver (2005). "Patterned Assembly of Genetically Modified Viral Nanotemplates via Nucleic Acid Hybridization." Nano Letters **5**(10): 1931-1936.

Yildiz, I., K. L. Lee, K. Chen, S. Shukla and N. F. Steinmetz (2013). "Infusion of imaging and therapeutic molecules into the plant virus-based carrier cowpea mosaic virus: cargo-loading and delivery." J Control Release **172**(2): 568-578.

Yin, P. T., S. Shah, M. Chhowalla and K.-B. Lee (2015). "Design, Synthesis, and Characterization of Graphene–Nanoparticle Hybrid Materials for Bioapplications." Chemical Reviews **115**(7): 2483-2531.

Yokoo, N., T. Togashi, M. Umetsu, K. Tsumoto, T. Hattori, T. Nakanishi, S. Ohara, S. Takami, T. Naka, H. Abe, I. Kumagai and T. Adschiri (2010). "Direct and selective immobilization of proteins by means of an inorganic material-binding peptide: discussion on functionalization in the elongation to material-binding peptide." J Phys Chem B **114**(1): 480-486.

Yoshioka, K. (2013). "What is the benefit of computer-assisted image analysis of liver fibrosis area?" J Gastroenterol **48**(8): 996-997.

Zdanowicz, M. and J. Chroboczek (2016). "Virus-like particles as drug delivery vectors." Acta Biochim Pol **63**(3): 469-473.

Zeltins, A. (2013). "Construction and Characterization of Virus-Like Particles: A Review." Molecular Biotechnology **53**(1): 92-107.

Zhang, X.-Q., X. Xu, N. Bertrand, E. Pridgen, A. Swami and O. C. Farokhzad (2012). "Interactions of nanomaterials and biological systems: implications to personalized nanomedicine." Advanced drug delivery reviews **64**(13): 1363-1384.

Zhao, Q., M. J. Allen, Y. Wang, B. Wang, N. Wang, L. Shi and R. D. Sitrin (2012). "Disassembly and reassembly improves morphology and thermal stability of human papillomavirus type 16 virus-like particles." Nanomedicine **8**(7): 1182-1189.

Zheng, L., P. J. Wayper, A. J. Gibbs, M. Fourment, B. C. Rodoni and M. J. Gibbs (2008). "Accumulating variation at conserved sites in potyvirus genomes is driven by species discovery and affects degenerate primer design." PLoS One **3**(2): e1586.