POLITÉCNICA

UNIVERSIDAD
POLITÉCNICA
DE MADRID

UNIVERSIDAD POLITÉCNICA DE MADRID
ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA
AGRONÓMICA, ALIMENTARIA Y DE BIOSISTEMAS

BIOTECHNOLOGICAL ENGINEERING OF TURNIP MOSAIC VIRUS-DERIVED NANOPARTICLES BY FUNCTIONALIZATION WITH NATURAL COMPOUNDS OF DIFFERENT BIOLOGICAL ACTIVITIES

TESIS DOCTORAL
EDITH VELÁZQUEZ LAM
ING. EN BIOTECNOLOGÍA

2021

Instituto de
Innovación y
Transferencia
de Tecnología
Gobierno de Nuevo León

INIA
Instituto Nacional de Investigación
y Tecnología Agraria y Alimentaria

CBGP
CENTRO DE BIOTECNOLOGÍA
Y GENÓMICA DE PLANTAS
UPM-INIA

EXCELENCIA
SEVERO
OCHOA

CAMPUS
DE EXCELENCIA
INTERNACIONAL

UNIVERSIDAD POLITÉCNICA DE MADRID

02. Escuela Técnica Superior de Ingeniería

Agronómica, Alimentaria y de Biosistemas

“Biotechnological engineering of Turnip mosaic virus-derived nanoparticles by functionalization with natural compounds of different biological activities”

AUTORA:
Edith Velázquez Lam
Ing. en Biotecnología
2021

UNIVERSIDAD POLITÉCNICA DE MADRID

02. Escuela Técnica Superior de Ingeniería

Agronómica, Alimentaria y de Biosistemas

TESIS DOCTORAL:

“Biotechnological engineering of Turnip mosaic virus-derived nanoparticles by functionalization with natural compounds of different biological activities”

AUTORA:

Edith Velázquez Lam

Ing. en Biotecnología

DIRECTOR:

Fernando Ponz Ascaso

Doctor en Bioquímica y Biología Molecular

Madrid, 2021

TRIBUNAL

Presidente: Jose Manuel Palacios Alberti

Secretaria: Aurora Fraile Pérez

Vocal: Laura Benítez Rico

Vocal: María Corina Lorz López

Vocal: Pablo Lunello Lindow

Suplente 1: Miguel Regules Aranda

Suplente 2: Fernando Larcher Laguzzi

ACKNOWLEDGMENTS

I would like to express my deepest appreciation to my advisor Dr. Fernando Ponz. I'm truly grateful for his patience, continuous support throughout my PhD research, motivation and knowledge. Thank you for inspiring me to strive for the best.

Thanks to Dr. Jose Manuel Palacios Alberti, Dr. Aurora Fraile Perez, Dr. Laura Benítez Rico, Dr. María Corina Lorz López and Dr. Pablo Lunello Lindow for accepting the invitation of being my committee in this thesis.

Also, thanks to INIA and CBGP for providing me a place and resources to do my PhD research.

I would also like to express my gratitude to Dr. Jorge Moreno Cuevas, Dr. Mario Alvarez and Dr. Grissel Trujillo in Tecnológico de Monterrey, who provided me an opportunity to join their teams for my internships. Without their support it wouldn't be possible to conduct part of this research.

My sincere appreciation to Dr. Juan Imperial, Dr. Carmen Segrelles, Dr. Emilia Lopez Solanilla, Dr. Saray Santamaría Hernandez, Dr. Araceli Diaz, Dr. Jaime Tomé, Dr. Juan Carlos Saiz and Dr. Estela Escribano for your kindness, support, patience and wisdom in the different experimental phases within this thesis.

Also, big thanks to CONACYT and the Institute of Innovation and Technological Transfer of Nuevo León that gave me the scholarship which made possible for me to do my PhD studies in Madrid and provided the funds for me to return to Monterrey to do my internships. Their support means the world to me and I'll do my best to continue doing good science and contribute to society by applying everything that I learned here.

Thanks to my lab mates that have not only supported me throughout the years but, made this process a whole more enjoyable. Luci, Carmen, Silvia, Deivid, Irene amongst many others that passed through the lab, you have given me valuable lessons that I will carry on with joy.

To my friends-family, Ivonne, Nelly, Cynthia, Ana (Monterrey), Jorge, Rafa, Milla, Miguel, Julian, Rodrigo, Ana (Madrid), Fran, Guille, Montse, Iciar and Bea thank you for your continuous support, great memories and being an important backbone in my life throughout these years. Thalles, thank you for everything, your continuous support and love have meant the world to me. I'm lucky to have so many amazing people in my life. Finally, thanks to my parents to whom I owe everything. There are no words that could ever express how much I appreciate your love, support and making me believe in myself.

Everyone mentioned here has contributed to the fulfillment of this thesis and is because of you this was possible. My deepest gratitude.

ABBREVIATIONS

7-AAD	7-aminoactinomycin D
AFM	Atomic force microscopy
BSA	Bovine serum albumin
CP	Capsid
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
FBS	Fetal bovine serum
GelMA	Gelatin methacryloyl
MOI	Multiplicity of infection
NP	Nanoparticle
ORF	Open reading frame
PapMV	Papaya mosaic virus
PFU	Plaque forming units
PsPto	<i>Pseudomonas syringae</i> pv. strain DC3000
PVX	Potato virus X
TEM	Transmission electron microscopy
TMV	Tobacco mosaic virus
TuMV	Turnip mosaic virus
ULA	Ultra-low attachment
VLPs	Virus-like particles
VNPs	Viral nanoparticles
ZIKV	Zika virus

INDEX

TRIBUNAL	V
ACKNOWLEDGMENTS	VII
ABBREVIATIONS	IX
INDEX	XI
RESUMEN	XV
ABSTRACT	XVII
INTRODUCTION	1
1.1 Nanobiotechnology	1
1.2 Viral nanoparticles	5
1.3 Potyvirus and Turnip mosaic virus	8
1.4 Genetic functionalization of TuMV VNPs	10
1.5 Chemical conjugation for VNP functionalization	11
OBJECTIVES	15
RESULTS	19

CHAPTER 1: CHARACTERIZATION OF TuMV AS A TECHNOLOGICAL PLATFORM	21
1.1 Introduction	23
1.2 Experimental section	26
1.3 Results and discussion	31
1.4 Conclusions and perspectives	38
CHAPTER 2: FUNCTIONALIZATION OF TuMV DERIVED VNP _s WITH PLANT POLYPHENOLS	39
2.1 Introduction	41
2.2 Experimental section	51
2.3 Results and discussion	61
2.4 Conclusions and perspectives	86
CHAPTER 3: BIONANOTECHNOLOGY FOR TISSUE ENGINEERING: DEVELOPMENT OF GELATIN METHACRYLOYL (GELMA) ENRICHED WITH VIRUS NANOPARTICLES (VNPS).	89
3.1 Introduction	91
3.2 Experimental Section	93
3.3 Results and discussion	97
3.4 Conclusions	110
GENERAL DISCUSSION	113

Evaluation of TuMV biotechnological applications	115
Functionalization of TuMV derived VNP_s with plant polyphenols	116
Bionanotechnology for tissue engineering: development of gelatin methacryloyl (GelMA) enriched with TuMV-derived nanoparticles.	118
CONCLUSIONS	121
REFERENCES	125

RESUMEN

En la presente tesis se evalúan nanopartículas de plantas derivadas del Virus del mosaico del nabo (TuMV) como plataformas tecnológicas. Estas nanopartículas virales (VNPs) pueden ser modificadas químicamente al conjugar moléculas a la superficie de la cápsida del virus. Lo anterior permite diseñar nanopartículas que responden a un número de necesidades, lo que las hace un biomaterial invaluable. Las VNPs fueron producidas al inocular mostaza india (*Brassica juncea* L.). Se analizó el potencial de TuMV para aplicaciones biomédicas evaluando su citotoxicidad en cultivos celulares. Se conjugaron químicamente las moléculas fluorescentes Alexa₅₅₅ y Cy_{5.5} para determinar la interacción de TuMV con células de mamífero. Se encontró que TuMV tiene interacción con las células en la parte exterior de la membrana plasmática y se encontraron evidencias de la presencia de TuMV en el espacio intracelular. Se inyectó TuMV-Cy_{5.5} a ratones inmunodeficientes Foxn1^{nu}, se hizo un seguimiento *in vivo* durante cuatro días, se sacrificaron los animales y se recogieron muestras de tejido para determinar la distribución de TuMV en órganos. Se encontró que las nanopartículas se acumulaban en el hígado y en tejido de tumor lo que abre la puerta para el uso de TuMV como agente terapéutico.

En esta tesis, la conjugación química de galato de epigallocatequina (EGCG) se llevó a cabo mediante la reacción de Mannich. Se encontró que TuMV-EGCG mostraba actividades antimicrobiana, antibiofilm y antitumoral superiores, en comparación con EGCG libre. Los ensayos *in vivo* de *Pseudomonas syringae* en plantas de tomate mostraron que EGCG-TuMV es capaz de disminuir la virulencia bacteriana. En cuanto a la actividad antiviral, se encontró que EGCG-TuMV mostraba un menor efecto en la inhibición de infección del virus Zika (ZIKV).

Finalmente, se evaluó TuMV como aditivo para gelatina metacrilada (GelMA). Los hidrogeles han cobrado importancia debido a que tienen cualidades similares a la matriz extracelular de mamíferos y se han convertido en un biomaterial clave para la ingeniería de tejidos. Los ensayos con formulaciones de TuMV-GelMA mostraron su compatibilidad como biotinta para impresiones tridimensionales que pueden ser usadas como soporte para cultivos celulares. El factor de crecimiento epitelial (EGF) se conjugó químicamente a la superficie de TuMV y se generaron formulaciones EGF-TuMV-GelMA. Se encontró que EGF retiene su actividad y que su adición a la matriz de gel estimula la proliferación celular. En este trabajo se pone de manifiesto la versatilidad de TuMV como herramienta tecnológica. Es posible modificar la superficie de la cápsida con diferentes moléculas con actividades biológicas diferentes manteniendo su actividad. Lo anterior reafirma su potencial para numerosas aplicaciones biotecnológicas.

ABSTRACT

In this thesis, plant-nanoparticles derived from *Turnip mosaic virus* (TuMV) were evaluated as a technological platform. These Viral Nanoparticles (VNPs) can be modified by chemically attaching molecules to its surface in order to cater different purposes, which make them valuable biomaterials. The VNPs were produced by inoculation of Indian mustard (*Brassica juncea* L.). TuMV was evaluated for biomedical applications by testing cytotoxicity to cell cultures. Furthermore, TuMV was coupled with fluorescent molecules Alexa₅₅₅ and Cy_{5.5} to assess interaction of TuMV with cells. Interaction of TuMV with mammalian cell lines was found at both extracellular and intracellular levels. TuMV-Cy_{5.5} was injected to immunodeficient mice (Foxn1^{nu}), allowed to distribute *in vivo* for four days, and organs were harvested to determine organ distribution of VNPs. It was found that liver and tumor tissue showed the highest accumulation of TuMV-Cy_{5.5}.

In this thesis, the chemical conjugation of epigallocatechin gallate (EGCG) was performed via Mannich reaction. TuMV-EGCG showed enhanced antimicrobial, antibiofilm and antitumor activities in comparison to free EGCG. *In vivo* assays of *Pseudomonas syringae* against tomato plants showed that EGCG-TuMV was capable of diminishing bacterial virulence. Also, antiviral activity against Zika virus (ZIKV) was tested finding low antiviral activity.

TuMV was tested as an additive for gelatyn methacryloil (GelMa). Hydrogels resemble some of the properties of the extracellular matrix in mammals and have become a key biomaterial in the tissue engineering arena. Assays with TuMV-GelMA formulations showed that this can be used as a bioink for 3-D printing and as a scaffold for cell proliferation enhancing elongation. Furthermore, the epithelial growth factor (EGF) was chemically attached to TuMV surface and used as a functionalized additive for GelMa bioinks. Tests showed that EGF retained its activity and its addition to a gel matrix allowed cellular proliferation.

In this thesis, the possibility of chemically attaching different molecules while retaining its activity is proven. Also, it tests the versatility of TuMV as a biomaterial that can be used in an array of different scenarios.

INTRODUCTION

1.1 Nanobiotechnology

The interest in developing materials and tools at the nanometer scale has been increasing over recent years. Nanobiotechnology has an impact in a multitude of disciplines with a large potential for boosting technological developments. Nanoscale engineering offers new ways to prevent, detect and treat illnesses (Koudelka *et al.*, 2015). It offers the development of new materials for plant protection and innovative techniques for bioremediation (Gogos, Knauer and Bucheli, 2012; Tripathi *et al.*, 2018). Also, it produced a revolution on the food industry since it has been applied in encapsulation, flavor enhancement, storage improvement amongst others (Chellaram *et al.*, 2014).

By nature, this is a multidisciplinary field in which different areas of science have to converge with biology in order to exploit chemical, physical and biological properties of materials. From a biological point of view, this is particularly interesting since nanomaterials are compatible with the dimensions of naturally occurring functional units, thus enabling more effective interactions (Buzea, Pacheco and Robbie, 2007). Size is not the only advantage of nanotechnology; nanomaterials can be based on organic or inorganic materials or can be presented as hybrid structures. Different materials present distinct characteristics that can be manipulated in order to have different configurations for different purposes. The surface of a nanoparticle

(NP) can be modified, which allows a high concentration of a molecule of interest (Naito *et al.*, 2018). Also, the hollow structure of some NPs allow to introduce other molecules in order to protect them from the environment or avoid undesired interactions until they reach their target (Sánchez-Navarro and Rojo, 2012).

The variety of different materials in combination with the increasing interest in the field have allowed thousands of patent applications since 2000 (Moeinzadeh and Jabbari, 2017). The increasing use of NPs has raised concerns about possible effects on the environment and human health. In order to address this subject, it is important to take in consideration the nature of the material used. NPs can be classified in 4 different material-based categories: carbon, inorganic, organic, and composite (Jeevanandam *et al.*, 2018). Each material has a number of advantages and disadvantages to evaluate, depending on the application addressed.

Carbon-based nanomaterials, as the name describes, contain carbon and are found in morphologies like spheres, hollow tubes and ellipsoids. They have some advantages such as their chemical inertness, easy functionalization, high resistance to photobleaching and large surface area (Patel, Singh and Kim, 2019). On the other hand, they have disadvantages such as cost or poor batch-to-batch reproducibility (Scida *et al.*, 2011). Though this type of material might sound promising, the possible effect of the environment is hard to predict. Analytical measurements and mathematical modeling need to improve in order to determine a possible outcome in a complex environment as the application of carbon-base NPs is increasing (Mottier *et al.*, 2017). Also, the cytotoxicity of this type of material remains controversial. Some reports have shown that there is cytotoxicity against a number

of cell lines while others report exactly opposite results which is also a direct result of the state-of-the-art of analytical techniques (Yuan *et al.*, 2019).

In the inorganic-based nanomaterials field most of reported nanomaterials are made of metals or metal oxides. This is a wide classification whose members offer unique features that are attractive for various purposes. Noble metal NPs characterize themselves by their unique plasmon resonance (Anselmo and Mitragotri, 2015). Amongst these, gold is the most intensively researched. Gold NPs are biocompatible, have a strong photoelectric absorption coefficient, exhibit natural antimicrobial activity, and are relatively simple to produce and modify (Holzinger, Goff and Cosnier, 2014). Although these characteristics seem attractive, size distribution, crystallinity and homogeneity in chemical composition, and producing methods still need improvement. In the case of metallic NP production, there is a need for developing cleaner, less polluting synthetic routes that still preserve the positive qualities of the NP (Hartmann *et al.*, 2012). Also, environmental impact of inorganic NPs is an issue since reports have stated that there is a wide array of ways in which they might cause toxicity, industrial production of these NPs have leaked them into the environment where bioaccumulation has become a problem (Gupta and Xie, 2018).

Organic-based nanomaterials are made of organic matter. They are based upon natural or synthetic molecules. With the exception of dendrimers and other polymeric organic nanoparticles, this group of nanoparticles is formed by a number of organic molecules driven together either by chemical binding or self-organization. In contrast to their carbon and inorganic counterparts, it was found that organic-

based NPs degrade the fastest (Kümmerer *et al.*, 2011) which has led to believe that these might provide an alternative with less polluting effects.

Arguably, protein-based nanoparticles offer one of the most attractive subgroups in this NP classification. Not only they are biodegradable, they are also metabolizable and their characteristics allow them to be modified in a large number of ways (Weber *et al.*, 2000). Proteins are a versatile biomaterial since chemical and physical properties greatly vary with their amino acid composition. Unlike synthetic polymers, proteins have different functional groups for covalent and non-covalent chemical modifications. Although they offer advantages, there are some limitations to consider while evaluating possible applications. Composition and purity are difficult to control and non-autologous proteins can be immunogenic (Ziv, Avtalion and Margel, 2008). Also, the performance of particles made from protein are influenced by a number of factors such as solubility, purity, molecular weight and surface composition (Tarhini, Greige-Gerges and Elaissari, 2017). For example, surface properties such as charge and hydrophobicity/hydrophilicity have a direct impact on the level of adsorption in the field of drug delivery. A neutral, hydrophilic surface is preferable to avoid macrophage uptake (Brigger, Dubernet and Couvreur, 2002).

Protein-based NPs can be classified according to the source of the material: animal, microorganism or plant protein. Each subgroup possesses both advantages and disadvantages. Animal proteins have low toxicity but there is a risk of infection from pathogen contamination (Leo *et al.*, 1997). Plant-based proteins production requires less consumption of natural resources in comparison to that of animal-based proteins (Wan, Guo and Yang, 2015). Hydrophobic plant proteins have the capability

of producing carries able to slowly release their cargoes which are particularly interesting since they do not need chemical modifications, thus not requiring potentially toxic linkers (Chen, Remondetto and Subirade, 2006). For these reasons, the focus has shifted on studies related to bioactive compounds, chemical composition or pharmacological potential of plant products that have the potential of reducing side effects on health and the environment (Beutler, 2019).

1.2 Viral nanoparticles

Within nature, there is a plethora of nanostructures that can be used as biomaterials. Viruses are a prime example of these natural-occurring building for technological applications. They are ubiquitous within nature and their structure is symmetrical and monodisperse. This structure is usually comprised of a genomic nucleic acid content and a protective protein shell, named capsid, that is assembled by a limited number of identical proteins (Douglas, 2006). Viral capsids are a useful tool for different applications since they are easy to produce, capable of self-assembly, highly modifiable and biocompatible (Benjamin *et al.*, 2020).

The primary function of the protein shell is to protect its nucleic acid content. Because of this structure, viruses are able to withstand a wider range of pH and temperature. The capsid of viruses is assembled according with one of the two fundamental types of symmetry: helical and icosahedral (Gergerich and Dolja, 2006). This protein shell is of vital importance for viral nanoparticle (VNP) engineering since

the structure provides available sites for manipulation. It allows to load cargo on the interior of the capsid and/or modify the exterior for a number of purposes.

The versatility of VNPs is not limited to their structure, VNPs can be purified from their natural hosts or can be produced on heterologous expression systems (Pokorski and Steinmetz, 2011). VNPs can be produced with or without their genomic content. Virus-Like particles (VLPs) are made of empty viral capsids modified through biotechnology. Depending on the application, VLPs can be used to address safety issues over VNPs with genomic content but they have a clear disadvantage in the production process since characterization can be more difficult (Chattopadhyay *et al.*, 2017). The versatile nature of VNPs has allowed the development of various nanoparticles that are valuable in a wide array of areas from sensors (Bäcker *et al.*, 2017) to drug-delivery systems (Jobsri *et al.*, 2015).

Figure 1 | Schematic representation of virion and VLP.

Viruses can be modified either genetically or chemically. The repetition of a limited number of proteins makes possible to come up with a variety of modifications that allow their manipulation. The proteinaceous nature of VNPs allow for standard bioconjugation protocols to address available amino acids. The most common reactions used for this purpose involve side chains of lysine, cysteine, and aspartic/glutamic residues (Smith, Hawes and Bundy, 2013; Spicer and Davis, 2014). There are three surfaces that can be chemically or genetically modified for the production of novel nanomaterials: the interior, the exterior and the interface between subunits. The later allows to use nanoparticles in a wide variety of configurations and applications (Figure 2).

Figure 2 | Surfaces for functionalization and applications.

1.3 Potyvirus and Turnip mosaic virus

The *Potyviridae* family is comprised of eight genera of viruses. Members of this family are plant-infecting single-stranded positive-sense RNA viruses, with flexible, flexuous and filamentous virus particles (Revers and García, 2015b). This family has more than 200 species from which 168 are members of the *Potyvirus* genus (Wylie *et al.*, 2017). These viruses are non-persistent aphid transmitted, some of them can be seed-transmitted (Gadhav *et al.*, 2019). Potyviruses have been widely studied since they are plant-pathogens responsible for crop losses.

Potyvirus genomes contain a single open reading frame (ORF) that is translated into a single large protein (340-370 kDa) that is cleaved into at least 10 mature proteins by three viral-encoded proteinases (P1, HC-Pro, VPg-Pro) (Riechmann, Lain and Garcia, 1992; Valli *et al.*, 2014). The proteins that are translated from this ORF are described in Figure 4.

Figure 3 | Schematic diagram of the potyvirus polyprotein. Protein 1 (P1), helper component proteinase (HCpro), protein 3, protein 6 kDa number 1 (6K1), cytoplasmic-inclusion protein (CI), protein 6 kDa number 2 (6K2), nuclear-inclusion proteins a and b (NIa and NIB), genome-linked protein (VPg) and capsid protein (CP). PISPO has not been reported in all potyviruses. Gray proteins are produced in a different manner. (Yuste Calvo, 2019)

Turnip mosaic virus (TuMV) belongs to the Potyvirus genus and is spread by 40-50 species of aphids. The symptoms of infected plants include chlorotic local lesions, mottling, lamina becomes necrotic, puckering and rugosity (Moreno and Fereres, 2012). Its virions are flexuous, non-enveloped filaments with a diameter of 13.5 nm and a length of about 700 nm (Cuesta *et al.*, 2019). The capsid protein (CP) is a three-domain protein: core, amino and carboxyl domains. The amino domain is exposed on the virion surface and is the most variable domain amongst the members of the *Potyviridae* family.

CP plays an essential role in the functionalization of NPs (Sánchez *et al.*, 2013; Cárdenas-Vargas *et al.*, 2016; González-Gamboa *et al.*, 2017). Each virion is composed of approximately 2000 CP copies, 33 kDa in size. The structural characteristics of the potyvirus make TuMV a good candidate as a display system for molecules of interest.

1.4 Genetic functionalization of TuMV VNPs

The fact that the N-terminus domain is exposed to the environment makes peptide-fusion possible. In previous studies, the addition of a peptide in this domain does not affect viral infectivity (Ibort, 2012; Duval *et al.*, 2016). Maintaining infectivity (in virions) and self-assembly capacity after the genetic fusion of a peptide is key for assessing feasibility of genetic functionalization. The main factors that have an effect on the later are net charge, hydrophobicity, size and cysteine content of the peptide of interest (Li *et al.*, 2007; Bárcena and Blanco, 2013).

With the previous considerations in mind, genetic fusion is possible using the plasmid p35-Tunos-Vec01-NAT1 (Sánchez *et al.*, 2013). Chimeras are produced by cloning DNA corresponding to specific epitopes into the gene that encodes for CP. Upon assembly of the recombinant subunit proteins, the introduced peptides are presented on the VNP surface in a repetitive, ordered fashion.

Genetic functionalization is a strategy that has a number of advantages. Designing a vector and virion transformation has only to be made once. A successful NP design vector can be stored for later use and plants can be used as bio-factories.

However, it is also important to understand disadvantages. Though the structure of TuMV is known, predicting the effects of the inserted peptide over self-assembly and infectivity has proven to be difficult and time-consuming. Though genetic fusion has been successfully applied in the past, the trial and error approach cannot be avoided which makes it also time-consuming (Mateu, 2011). The size of the peptide has to be restricted to less than 20-30 amino acids with minimal cysteine content in order to avoid unwanted disulfide bonds. Finally, the addition of non-

proteinaceous molecules cannot be addressed by genetic fusion which limits not only the size but also the type of molecules that can be added via this strategy.

1.5 Chemical conjugation for VNP functionalization

Capsids are made of protein which provide sidechains that can be used as anchoring sites. This opens the doors for a wider array of possibilities in VNP design. Chemical conjugation not only gives the possibility to attach those molecules that cannot be approached by genetic fusion, but it also allows multiple functionalization of a VNP.

Chemical conjugation of VNPs can be achieved through various techniques like covalent coupling, click chemistry, or copper (I)-catalyzed azide-alkyne as shown in Figure 5. Small molecules have access to the viral inner cavity, which can be used as a nanotemplate or as a nanoreactor. The surface of the capsid is not only a great site for chemical modification but also shows a multivalent ligand display. Finally, the virus architecture interface provides additional ways to further manipulate the capsid structure.

Using chemical reactions to covalently attach a molecule to an available residue of the CP is aided by decades of advances in peptide chemistry. Lysine, cysteine, glutamic and aspartic acids, and tyrosine are known to provide reactive side chain moieties that can form biocompatible covalent bonds (Smith, Hawes and Bundy, 2013).

Lysine and cysteine are the more commonly used targets for VNP functionalization. Lysine ϵ -amino groups and protein N-termini provide primary amines which are reactive moieties. Predominantly, primary amines have been reacted with N-hydroxysuccinimidyl-esters (NHS-ester). Lysines are convenient conjugation sites since they are among the most common amino acids and also, since they are hydrophilic, often surface exposed (Gilis *et al.*, 2001). In addition, NHS-ester conjugation is a simple reaction and has wide commercial availability.

Cysteines can be targeted via maleimide-thiol reaction. Opposite to lysine, cysteine can be used for directed cross-linking as it only occurs at an estimated 1.1% (Gilis *et al.*, 2001). Maleimide linkers and custom synthesis are also commercially available.

In particular, TuMV nanoparticles have been successfully chemically modified using the aforementioned targets (Yuste-Calvo *et al.*, 2019). The later suggests for the potential of TuMV particles in VNP engineering.

Figure 4 | Basic conjugation chemistries on protein nanoparticles. (a) Addresses lysines and N-termini. NHS-ester conjugation (right) and thioimide conjugation (left). (b) Addresses cysteines. Maleimides are used for covalent bonding. (c) Addresses glutamic acid and aspartic acid. (d) Addresses tyrosine (e) Addresses present alkynes which react with azides in the presence of Cu(I). (f) Azides can undergo the Staudinger ligation (left), or Cu(I)-catalyzed click chemistry (e) or copper-free click chemistry (right). (g) Addresses tyrosine but remains orthogonal.

OBJECTIVES

The main aim of this study was to study the potential applications and functionalization of the *Turnip mosaic virus* virions. More specifically, the aims of the study were:

1. To determine thermal inactivation points of TuMV that do not modify its structure.
2. To determine cell toxicity and interaction of TuMV in mammal cell lines.
3. To determine *in vivo* distribution of TuMV in tumor-bearing immunodeficient mice.
4. To chemically conjugate polyphenolic compound epigallocatechin gallate (EGCG) to the surface of TuMV.
5. To evaluate the antitumor, antiviral and antimicrobial capacity of TuMV-EGCG nanoparticles.
6. To evaluate TuMV as an additive for gelatin-methacryloil (GelMa) for the engineering of a biomimetic material platform for cell attachment and proliferation.
7. To chemically conjugate Epidermal Growth Factor (EGF) to TuMV to the surface of TuMV.
8. To develop TuMV-EGF-GelMA formulations for cell attachment and proliferation.

RESULTS

CHAPTER 1: CHARACTERIZATION OF TuMV AS A TECHNOLOGICAL PLATFORM

CHAPTER 1: CHARACTERIZATION OF TuMV AS A TECHNOLOGICAL PLATFORM

1.1 Introduction

Advantages in nanotechnology have allowed the development of nanoscale materials that can be tailored for technological applications.

Within reported plant viruses, a high variability of shapes, sizes, and nucleic acid content has been found. Plant viruses are naturally found in a both, icosahedral and helical symmetries (Figure 1.1). Their size ranges from nanometers to micrometers. For example, geminiviruses are made of a hemcapsid of 18 nm diameter and 30 nm length, while closteroviruses are helical, flexuous particles of about 13 nm of diameter and 2 μm of length (Byv, 2012; Hesketh *et al.*, 2018). Finally, while most plant viruses have RNA genomes, plant viruses with DNA genomes also exist. Both cases are also found as single-stranded and double-stranded (Liu and Chan-Park, 2010; Hull *et al.*, 2016).

The classic example of a helical virus is the Tobacco mosaic virus (TMV), which has 2130 protein subunits in its capsid that can be used as functionalization sites. A deeper understanding of particle architecture, biophysical properties, and molecular structure has allowed the application of plant viruses in the biomedical field. For example, filamentous viruses such as potato virus X (PVX) and papaya mosaic virus (PapMV) have been explored for tissue penetration, tumor imaging, and vaccines (Steinmetz, 2010; Shukla *et al.*, 2014; Thérien *et al.*, 2017).

Figure 1. 1 | **Examples of plant virus structures that have been used as viral nanoparticles.** Structural data for icosahedral viruses were obtained from <http://viperdb.scripps.edu/>, and structural. Data for TMV were obtained from <https://www.ncbi.nlm.nih.gov>. For PVX and TuMV, schematic representations of the virus particles are shown.

The interest in plant viruses has increased throughout the years since the focus has shifted from being mainly studied as pathogens to becoming technological platforms. Other advantages attributed to them are their ease of purification, high stability of their capsids, and the simplicity of the composition of the capsid since they are usually made of one or two proteins.

Since their hosts are plants, they are non-infectious to mammals, which is an important factor from the biosafety standpoint. Since virulence is not a risk factor for human health and they retain all advantages of protein-based nanoparticles, plant viruses are good candidates for biomedical and pharmaceutical chemistry

applications (Yildiz, Shukla and Steinmetz, 2011; Grasso *et al.*, 2013; Singh *et al.*, 2016; Rohovie, Nagasawa and Swartz, 2017).

Arguably one of the most attractive characteristics of plant viruses as nanoparticles is their production yield. High viral load, with their ease of purification in comparison to their mammal counterpart, allows higher production in a shorter time (Ibrahim, Odon and Kormelink, 2019). For this reason, plant-based expression systems (molecular farming) has been established for large-scale protein expression. Plants are robust, inexpensive to grow, and have lower or no risk of endotoxin or mammalian pathogen contamination (Lico, Chen and Santi, 2008). Also, this system provides a technological platform that can be adjusted rapidly, with minimal manufacturing costs (Marsian and Lomonossoff, 2016).

In specific, protein-based nanomaterials such as VLPs, are a promising subclass of nanocarriers for drug delivery since they have demonstrated effectiveness in applications such as drug delivery (Shukla *et al.*, 2013; Wen and Steinmetz, 2016; Finbloom *et al.*, 2018; Pitek *et al.*, 2018). There are a number of advantages of VLPs over other synthetic materials. Unlike polymeric micelles and liposomes, this subclass of NPs have homogeneous size distribution and are relatively inexpensive to produce (Dedeo, Finley and Francis, 2011). VLPs have also demonstrated better cytotoxicity profiles and are degradable on biological conditions (Shukla *et al.*, 2013; Czapar and Steinmetz, 2017).

1.2 Experimental section

Production and purification of TuMV VNPs. TuMV (isolate UK 1) was propagated in Indian mustard, which were harvested 30 days post-inoculation. VNPs were purified from plant material, as previously described (Sánchez and Ponz, 2018). Plant tissue was finely ground at 4 °C, and a volume of chloroform at 4 °C was added for extraction. Phases were separated by centrifugation; the aqueous phase was filtered through Miracloth (MerckTM, Germany). VNPs were precipitated with 6% PEG 6000 (w/v) and 4% NaCl (w/v), and stirred for 90 min at 4 °C. Then, particles were recovered by centrifugation for 10 min at 12 000 g. The supernatant was discarded, and the pellet was resuspended in 0.5 M potassium phosphate, pH 7.5, and 10 mM ethylenediaminetetraacetic acid (EDTA) overnight at 4 °C. The resulting suspension was clarified by centrifugation for 10 min at 9 000 g. Then the VNPs were separated from the supernatant by centrifugation (2 h at 80 000 g). The resulting pellet was resuspended in 0.25 M potassium phosphate, pH 7.5, and 10 mM EDTA, and CsCl was added to a final density of 1.27 g/cm³. Then, the solution was centrifuged for 150 000 x g for 18 h at 4 °C. The band from the resulting gradient was recovered by punching the plastic tube with a needle and a syringe. The recovered band was diluted with 0.25 M potassium phosphate, pH 7.5, 10 mM EDTA, and centrifuged for 2 h at 80 000 x g. The resulting pellet was resuspended in 50% glycerol (v/v), 5 mM Tris, pH 7.5, 5 mM EDTA at a final concentration of 1 mg/ml and stored at -20 °C until future use.

Evaluation of EGCG-TuMV infectivity in *Chenopodium quinoa* after temperature treatment. Solutions of TuMV (1 mg/ml, 10 mM HEPES pH 7.2) were incubated in a water bath at different temperatures and times (Table 1).

Temperature (°C)	Time of incubation (min)
40	25
40	30
50	15
50	20
60	10
60	15

Table 1. 1 | List of temperatures and time of incubation for TuMV in 10 mM HEPES pH 7.2.

For assessing TuMV structural changes after temperature treatments, samples were examined by transmission electron microscopy (TEM). Electron microscopy grids (400-mesh nickel, carbon-coated) were placed on top of a drop of VNP solution and incubated for 10 min at room temperature, and washed with 50 mM borate buffer, pH 8.1. Then, microscopy grids were stained with 2% uranyl acetate. Finally, samples were examined on a transmission electron microscope (JEM 1400).

For assessing the infectivity of EGCG-TuMV after temperature treatment, groups of six three-week-old plants of *Chenopodium quinoa* were inoculated. Plants were dusted with carborundum, and two leaves per plant were manually inoculated with

5 µl of the corresponding solution. Plants were observed for 12 days; symptoms were recorded.

Exposure to TuMV *in-vitro* assay. Caco-2 (Colorectal adenocarcinoma, ATCC® HTB-37™), A459 (lung carcinoma, ATCC® CCL-185™), MCF7 (mammary gland, ductal carcinoma, ATCC® HTB-22™), BT 474 (mammary gland, ductal carcinoma, ATCC® HTB20™), SKBR 3 (mammary gland, adenocarcinoma, ATCC® HTB-30™), SiHa (cervix, squamous cell carcinoma, ATCC® HTB-35™) and mesenchymal stem cells (mouse, donated by The Cell Therapy group, Tec de Monterrey) cells were cultured in plated in a 96-well microtiter plate and allowed to attach overnight in the growth medium. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin was used for cell culture of all aforementioned cells. After overnight incubation, the medium was aspirated, and each well was washed three times with PBS. Medium with TuMV (0.01 and 0.10 µg/µl) was added, and cells incubated for 24 hours. After the time of exposure of TuMV elapsed, cells were washed with PBS three times. For cell proliferation, the MTS assay CellTiter 96®One solution (Promega, United States) was performed according to the instructions from the manufacturer. After 24-hour incubation, the medium was aspirated, wells were washed three times with PBS. 100 µl serum-free medium and 20 µl of CellTiter solution were added. Cells were incubated at 37 °C, humidified 5% CO₂ atmosphere. Absorbance was recorded at 490 nm.

Conjugation of VNPs and fluorescent tags (AlexaTM FluorTM 555 and Cy5.5). TuMV nanoparticles were tagged with AlexaTM FluorTM 555 NHS Ester (Life technologies, United States) and Cy5.5 NHS Ester (Life technologies, United States). TuMV suspension buffer was changed for HEPES 10 mM pH 7.5 by ultracentrifugation (50 min at 50 000 g), the pellet was resuspended up to a concentration of 1 mg/ml. AlexaTM FluorTM 555 or Cy 5.5 reagent was added in a 3:1 fold. Excess was removed by ultracentrifugation (50 min at 50 000 g) and resuspended with HEPES 20 mM pH 7.5 at a concentration of 1 mg/ml TuMV. TuMV-Alexa₅₅₅ and TuMV-Cy5.5 solutions were stored at 4 °C in the dark until further use.

Exposure to TuMV microscopy assay. 50000 Caco-2 cells, 16000 MCF7, MCF10A (mammary gland, ATCC® CRL-10317TM), and HeLa cells were seeded on a 24-well plate over glass covers. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin was used for cell culture of Caco-2, MCF7, and HeLa cells. For cell culture of MCF10A, DMEM-F12 supplemented with 5% FBS, 20 ng/ml epidermal growth factor (Agrenvec, Spain), 10 µg/ml insulin (Sigma-Aldrich, Germany), 0.5 µg/ml dehydrocortisone (Sigma-Aldrich, Germany), 100 ng/ml cholera toxin (Sigma-Aldrich, Germany), and 1% (v/v) penicillin-streptomycin was used. After overnight incubation, the medium was aspirated, and each well was washed three times with PBS. Corresponding growth medium with TuMV-Alexa₅₅₅ (0.025 µg/ µg) was added, and cells incubated for 3 and 24 hours. After the three-hour incubation, cells were washed thrice with PBS. Growth medium was added and incubated overnight. After the 24-hour incubation period elapsed, all cells

were treated with CellMask™ Deep Red Plasma Membrane stain (Thermo Fisher, United States), and DAPI (nuclei stain). Glass covers were mounted with ProLong™ Gold (Thermo Fisher, United States). Cells that were observed using a confocal fluorescence microscope (Leica TSC-SP8).

Cytometry assay for cell exposure to TuMV. 200000 MCF7 cells were cultured in plated in a 6-well microtiter plate and allowed to attach overnight in the growth medium. Cells were washed three times with PBS and medium with TuMV-Cy5.5 (0.025 µg/ µg) or no supplement was added, and cells incubated for 24 hours. After treatment, incubation cells were washed three times with PBS. Cells were detached, gently scratching the bottom of each well. Cytometry assays were performed on the Accuri™ C6 cytometer.

In vivo distribution of TuMV. Xenotransplant of 5000000 Cal33 cells (Tongue, Squamous Cell Carcinoma) in 1:3 Matrigel™ was injected to immunodeficient mice Foxn1^{nu}. Tumors were allowed to grow for four weeks. 100 µl of TuMV-Cy5.5 (1.5 µg/ µl) was applied to each mouse via retro-orbital injection (intravenous). Tumors were assessed *in vivo*, and fluorescence was measured using an Xtreme *In Vivo* imaging system (Bruker Biospin, Ettlingen, Germany). The rib cage, heart, tumor, spleen, kidney, fat, liver, lung, ganglion, small intestine, and leg were harvested, and fluorescence was measured.

1.3 Results and discussion

Heat treatment on TuMV has an effect on both viral structure (Figure 1.2A) and infectivity (Figure 1.2B). Incubating TuMV at 50 °C for 15 minutes hindered infectivity while largely maintaining virion structure. This is consistent with prior reports in which it has been found that heat inactivation of TMV-RNA causes hydrolysis of single sugar-phosphate bonds (Price, 1964). This information might be useful for further studies in which infectivity or RNA presence might be an issue for technological applications.

Figure 1. 2| TuMV infectivity assay (a) TEM Electron microscopy (TEM) micrographs of TuMV treated at 40 °C (25 and 30 min), 50 °C (15 and 20 min), 60 °C (10 and 15 min) (b) Plant infection symptoms of plants inoculated with pre-treated TuMV at different temperatures and times.

In order to assess the possible effects of TuMV on mammalian cell lines, different cell lines were exposed overnight to different concentrations. As seen in Figure 1.3, tested concentrations of TuMV in growth media did not have a negative effect on tested cell lines. Even though to our knowledge, the evaluation of TuMV for biomedical applications is yet to be determined, the lack of evidence of acute cell toxicity is not a surprise since other plant-filamentous-virus-derived nanoparticles have been evaluated with similar results (Shukla *et al.*, 2014). This is key since it opens the possibility of exploring the biomedical application of TuMV.

Figure 1. 3 Cell proliferation test after TuMV overnight exposition. Cells were exposed to different concentrations (0.01 and 0.10 µg/ µl) of TuMV. Percentages are calculated in relation to their respective untreated control. Data represent the means and standard errors of three replicates.

To determine possible interactions of TuMV with different cell lines, *in vitro* cell cultures were analyzed by confocal microscopy after exposure to TuMV-Alexa₅₅₅. For all tested cell lines, there was a clear interaction of the cells with TuMV-Alexa₅₅₅ (Figure 1.4). According to the 3-D surface plots, TuMV-Alexa₅₅₅ are seen at the surface of the cells in both treatments (3 and 24 hours). The further assessment indicates that there is some level of internalization of TuMV-Alexa₅₅₅ (yellow zones in 3-D surface plots and Figure 1.5). Finally, flow cytometry assays with MCF7 cells confirmed that TuMV is able to interact with cells (Figure 1.6); 85% of the population of exposed cells were tagged with TuMV-Alexa₅₅₅.

Cellular interactions might be useful for the development of therapeutic carriers and diagnostics tools. The application of plant virus-derived nanoparticles can also represent an advantage in strategies that were developed and proven to work effectively for rods such as carbon nanotubes (Liu *et al.*, 2008).

The organ biodistribution for TuMV-AlexaCy_{5.5} was determined by following the injection of 100 µl of 1.5 mg/ml solution of TuMV-AlexaCy_{5.5} in HEPES pH 7.2 into retro-orbital of tumor-bearing and one healthy Foxn1^{nu} mice, which were sacrificed four days post-administration. Heart, tumor, spleen, kidney, fat, liver, leg, small intestine, ganglion, lung, and rib cage were dissected and analyzed using Xtreme *In Vivo* imaging system (Figure 1.7). As seen in Figure 1.7a(ii), TuMV-AlexaCy_{5.5} decayed over time, which is an indication of tissue clearance. Mouse #2 presented a constant slow of signal decay while tumor-bearing mice presented a different behavior, which is consistent with tumor homing.

Organ distribution of TuMV (Figure 1.7b) showed that TuMV is deposited in the liver and tumor tissue. These findings are consistent with previous studies with PVX (Shukla *et al.*, 2013). There is evidence that filamentous nanomaterials are suitable for drug delivery. Non-spherical materials show a better capacity for tumor homing, and in specific, elongated materials present ligands more effectively to target cells. The obtained results suggest the viability of TuMV as fluorescent probes.

Figure 1. 4| Evaluating TuMV-Cell interactions using confocal microscopy. Caco-2, HeLa, MCF7 and MCF10A cells were exposed to 0.025 mg TuMV-Alexa₅₅₅/ml medium. Cell nuclei were stained with DAPI and are shown in blue. Plasma membranes were stained with CellMask deep red. TuMV-Alexa₅₅₅ is shown in green. Imaging was performed using a Leica TCS-SP8 confocal microscope. Images were created using Image J.

Figure 1. 5 | Evaluating TuMV-Cell interactions using confocal microscopy. HeLa and MCF7 cells were exposed to 0.025 mg TuMV-Alexa₅₅₅/ml medium. Cell nuclei were stained with DAPI and are shown in blue. Plasma membranes were stained with CellMask deep red. TuMV-Alexa₅₅₅ is shown in green. Imaging was performed using a Leica TCS-SP8 confocal microscope. Images were created using Image J.

Figure 1. 6 | Evaluating TuMV-cell interactions using flow cytometry assay. MCF7 cells were exposed to 0.025 mg TuMV-Alexa₅₅₅/ml medium overnight. Histograms: (a) cells only, (b) cells exposed to TuMV-Cy5.5 (c) histogram overlay of a and b.

Figure 1. 7 | *In-vivo* distribution of TuMV. [a] (i) *In vivo* imaging of mice injected with TuMV Cy5.5 (1.5 mg/ml). Control was not treated with TuMV. (ii) Signal intensity of mice tracked *in vivo* for 4 days. #2 healthy mouse, #3-6 tumor-bearing mice, and control tumor-bearing mouse untreated. [b] (i) Organ distribution of TuMV-Alexa₅₅₅ 4 days after administration. (ii) Schematic representation of sample position. (iii) Quantitative data of organ distribution of TuMV-Alexa₅₅₅ 4 days after administration. (c) (i) Qualitative data of tumor samples. (ii) Quantitative data of tumor samples.

1.4 Conclusions and perspectives

Temperature treatment for short periods proved to be efficient at 50 °C for 15 minutes. This can be useful when infectivity may be an issue. Future studies to assess RNA integrity and content should be performed.

We evaluated the suitability of fluorescence-labeled TuMV particles as probes for optical imaging applications. Since there was no evidence for acute cytotoxicity, the *in vitro* assays showed that TuMV is suitable for applications with human cells. There is a visible interaction in the plasma membrane, and TuMV seems to enter the cell, which is also a behavior expected from nanoparticles with this architecture.

The *in vivo* assay suggests that TuMV-Cy_{5.5} can be used as a fluorescent probe. Also, the signal of these nanoparticles decays over time, which is the expected behavior for proteinaceous nanoparticles. After injection, TuMV-Cy_{5.5} are concentrated in the liver and in tumor tissue. The latter is particularly interesting since functionalization with anticancer therapeutic agents can be explored. Nonetheless the approach to use TuMV as an optical imaging probe is worth exploring since it can provide a technological platform with high sensitivity and spatial resolution while avoiding exposure to radiation.

CHAPTER 2: FUNCTIONALIZATION OF T_uMV DERIVED VNP_s WITH PLANT POLYPHENOLS

CHAPTER 2: FUNCTIONALIZATION OF TuMV DERIVED VNP_S WITH PLANT POLYPHENOLS

2.1 Introduction

Polyphenols are substances that have an aromatic ring with one or more hydroxyl substituents and are ubiquitous within nature (Babu and Liu, 2009a). These compounds modulate the activity of a wide variety of enzymes and cell receptors (Middleton, Kandaswami and Theoharides, 2000). In plants, these secondary metabolites are often involved in the protective response to different stresses such as ultraviolet radiation and defense against pathogens (Baião *et al.*, 2017). The interest in polyphenolic compounds has been increasing throughout the years since they have been linked in protection against cardiovascular diseases, cancer, diabetes, microbial and viral infections (Pandey and Rizvi, 2009).

Polyphenols are classified based on the number of phenol rings and the structural elements binding them. The main groups of these compounds are phenolic acids, stilbenes, lignans, and flavonoids (Figure 2.1) (Niedzwiecki *et al.*, 2016). All prior subclasses have been linked to health-promoting activities from which flavonoids are the largest subclass amongst them, with more than 8000 identified compounds (Babu and Liu, 2009b).

Figure 2. 1 | Classes and subclasses of polyphenols. (Babu and Liu, 2009c; Tsopmo, Awah and Kuete, 2013; Simpson and Amos, 2017)

Functionalization of TuMV derived VNPs with EGCG for antimicrobial applications

Microbial control is an essential issue since it has economic and health impacts. The complexity of tackling this issue resides on the different aspects that need to be considered, such as the multiple mechanisms for microbial survival, the lack of novel antimicrobial molecules, or the changing regulations for antimicrobial agents because of increasing environmental awareness. All these aspects together cause reduced effectiveness of current approaches for attacking infections, which leads to economic, ecological, and life losses. This reality marks the increasing need for novel, potent agents to replace or boost existing antimicrobials (Coates *et al.*, 2002).

Resistance to antimicrobial agents happens naturally in bacteria and other microbes. Bacteria are capable of continually evolving, acquiring, and expressing resistance genes, which, ultimately, leads to the selection for resistant microbial clones (Pelgrift and Friedman, 2013).

Although antimicrobial resistance happens in nature, different reasons accelerate its development, making this issue harder to address. The misuse of antimicrobial drugs over time has led to the emergence and spread of multi-drug resistant bacteria. Also, there is a scarcity of newer drugs attributable to restrictive regulatory requirements (Aslam *et al.*, 2018).

Biofilm formation is another critical area to consider when dealing with antimicrobials and antimicrobial resistance. Bacterial biofilms are multicellular aggregates (Figure 2.2) that are usually attached to biotic and abiotic surfaces, in which bacteria are embedded in an extracellular matrix of self-produced polymers (Flemming *et al.*, 2016). Biofilms are ubiquitous and can form on various surfaces (Schofield and Wu, 2019). Bacteria within this structure usually display higher resistance to antimicrobials (Figure 2.2) than planktonic bacteria due to low penetration and accumulation of antimicrobials in the biofilm and conditions within the biofilm environment inactivate antimicrobials. As a result, bacterial biofilms are refractory to both antibiotic treatment and immune responses. Therefore, the biofilms of pathogenic bacteria play a crucial role in chronic infections.

Figure 2. 2 | (i) general structure of biofilm. (ii) antibiotic resistance due to biofilm formation.

The agricultural sector is also affected by microbial control since it is the source of losses in high-value crops (Johnson *et al.*, 2016). The increasing food demand, climate change, and regulatory limitations on plant protection products are aggravated by the emergence of new plant diseases (Lamichhane *et al.*, 2015). In specific, *Pseudomonas syringae* infects and attacks a wide range of agronomically important crops, including tomato, soybean, and olive (Rooney *et al.*, 2020). This type of bacteria lives in the foliage as an epiphyte. It is hard to tackle since it can spread rapidly because of the shortage of genetic diversity within commercial crops (Esquinas-Alcázar, 2005).

Current control measures involve the preventive use of traditional chemicals, copper compounds, or antibiotics that are, nonetheless, losing their efficacy due to bacterial resistance. Also, these conventional antimicrobial agents have

environmental effects that have to be considered. Traditional use of copper compounds raises concerns and source of debate since 95% of Cu released into the environment will accumulate in soil and aquatic sediments to potentially toxic levels ($> 50\text{--}500\text{ }\mu\text{g/ml}$) (Keller *et al.*, 2017). The need for new strategies for plant disease control is highlighted by the reasons above. Besides being effective, new methods should have minimal effects on the environment.

Plant-derived polyphenols have been reported to have antimicrobial and antibiofilm activities against a wide range of microorganisms, including bacterial, fungal, and viral pathogens (Hui *et al.*, 2017). In particular, studies have shown that the green tea polyphenolic flavonoids can inhibit the growth of a wide range of Gram-positive and Gram-negative bacterial species with moderate potency. (–)-Epigallocatechin gallate (EGCG), depicted in Figure 2.3, is the main flavonoid compound of green tea, comprising about 50% of the catechin pool (Taylor, Hamilton-Miller and Stapleton, 2005). Among the different flavonoids present in green tea, EGCG is the most bioactive and the most studied (Tauber, Schweiker and Levonis, 2020). A cup of green tea may contain 100–200 mg EGCG, while catechin and gallic acid are present in lower amounts (Gopal *et al.*, 2016).

Figure 2. 3 | EGCG molecular structure.

Although EGCG has been linked to a vast number of health-promoting activities, its commercial applications have been proven limited since it is sensitive to pH, temperature, and oxygen (Nadim *et al.*, 2014; Zeng *et al.*, 2017). For this reason, the design of a vehicle that maintains EGCG-attributed effects is an attractive strategy in order to overcome its lack of stability.

The incorporation of EGCG into NPs has been increasingly reported as a viable strategy to promote the biological activities of the compound. In specific, inorganic nanoparticles such as silver, copper, gold, and zinc oxide have been explored as carriers for antimicrobials (Chandra *et al.*, 2012; Sutradhar, Saha and Maiti, 2014; Suresh *et al.*, 2015; Chavva *et al.*, 2019). Although some of these materials have antimicrobial activities by themselves, metallic nanoparticles suffer from a number of limitations related to their environmental effects. Absorption of metals may have a negative impact on the structure and function of the photosynthetic apparatus (Tighe-Neira *et al.*, 2018). Toxicity has been linked to this type of NP; they tend to

accumulate in tissues and interact with proteins and DNA. Metallic nanoparticles are also known to produce stimulation of cytokines that contribute to cytotoxicity, immunotoxicity, and genotoxicity (Zazo *et al.*, 2017). Also, metallic NPs are prone to oxidation and aggregation and are challenging to extract and purify after green synthesis (Hulkoti and Taranath, 2014; Kruk *et al.*, 2015). Organic NPs lack the adverse effects of their metallic counterparts and have been developed as drug delivery carriers. Organic nanoparticles have been favored for biological applications; hence many organic nanoparticles have been tried as carriers for EGCG in different biological systems. These include lipidic (Smith *et al.*, 2010; Shtay *et al.*, 2019; Silva *et al.*, 2019), polysaccharide (Lei *et al.*, 2014; Chen *et al.*, 2019), or proteinaceous nanoparticles (Li *et al.*, 2014a; Malekhosseini *et al.*, 2019; Su *et al.*, 2020). Proteinaceous nanoparticles have received comparably lower attention, although casein-based nanoparticles have been explored, especially in the nutraceutical area (Xue *et al.*, 2014; Malekhosseini *et al.*, 2019). In one form or another, underlying reasons to try nanoparticle formulations for EGCG relate to the lack of stability of the compound in its free form.

Functionalization of TuMV derived VNPs with EGCG for antitumor applications

Cancer is a leading cause of death worldwide, which has been challenging to address since many factors influence its development. It is a continually evolving disease in which a myriad of molecular and genetic changes occurs. Carcinogenesis is a complex process in which multiple series of molecular and cellular modifications

ensue. In general, cancer development and progression can be summarized in three well-defined stages: initiation, promotion, and progression (Malarkey, Hoenerhoff and Maronpot, 2018).

Mortality due to cancer is projected to increase in the following years, and it has been estimated that by the year 2050, there will be 27 million new cases and 17.5 million deaths (Trosko and Kang, 2012). Conventional treatment for this disease includes surgery, chemotherapy, and radiotherapy (World Health Organization, 2014). The growing mortality rate from cancer, in combination with the adverse effects of the currently available treatments, has promoted the increasing search for new anticancer agents.

Plants have proven to be an excellent source of molecules that have shown promise in this search. In specific, plant polyphenols have shown suitable anticancer activities while having little to no adverse effects (Hastak *et al.*, 2003; Sakamoto *et al.*, 2013). Throughout the years, different studies have reported the antitumor activities of varying plant polyphenols (Habtemariam, 2002; Sánchez-Tena *et al.*, 2012) but the focus on the effort to understand the potential of polyphenols as anticancer therapeutic has been placed on EGCG.

EGCG is the most abundant polyphenol in green tea and has shown the potential for therapeutic applications in cancer. It has been reported that amongst the polyphenolic catechins found in green tea, this flavonoid has the most potent antiproliferative effects targeting biochemical and genetic functions that are unique to cancer cells (Hastak *et al.*, 2003; Du *et al.*, 2012). The chemoprotective activities of

EGCG have been observed on different phases of the cell cycle, which is one of the possible promising results found in pre-clinical studies.

A critical aspect of cancer development is cell proliferation, in which the cell is re-programmed, resulting in growth and progression (DeBerardinis *et al.*, 2008). EGCG has also proven to inhibit proliferation in the prostate (Albrecht *et al.*, 2008), bladder (Luo *et al.*, 2017), and breast (Wei *et al.*, 2018) cancer models, to name a few. Although the exact mechanism of action of this polyphenol over the cell remains unclear, it appears EGCG to affect cell-cycle regulatory proteins, leading to cell-cycle arrest and proliferation inhibition. Mainly it has been reported that EGCG induces G₀/G₁ cell-cycle arrest by inhibiting a number of proteins such as EGFR/cyclin D1 (MA *et al.*, 2014) or modulation of protein p53 (Huang *et al.*, 2009).

EGCG has also shown efficacy in the inhibition of angiogenesis, necrosis, motility, invasion, migration, and metastasis markers. In xenograft models, this catechin inhibited angiogenesis through the downregulation of vascular endothelial growth factor, hypoxia-inducible factor 1 alpha, and chemokine ligand 12 (Wang *et al.*, 2018). EGCG inhibition of migration, invasion, and motility of different cancer cell lines has been reported as the result of the inhibition of some proteins and downregulation of the expression of other pathways (Luo *et al.*, 2017; Rady *et al.*, 2018; Wei *et al.*, 2018).

Also, in contrast to other anticarcinogenic agents, data from clinical trials suggest that EGCG has a good safety profile with no adverse effects (Kumar *et al.*, 2016). Though this molecule has been widely explored, the extent of how EGCG interacts at the molecular level with the tumoral cells is yet to be fully understood. Nevertheless, reported data points to the promising potential of EGCG as a therapeutic agent.

Toxicity of EGCG is dependent on the dosing route of administration, where adverse induced effects by EGCG were reversible after cessation of treatment (Ramachandran *et al.*, 2016).

Despite the multiple promising results on EGCG as an alternative to chemotherapy, its applicability has proven limited. The lack of success in clinical settings relies on inefficient systemic delivery and bioavailability (Mereles and Hunstein, 2011). The stabilization of EGCG is yet to be characterized entirely, which is one of the challenges to improve the latter. This catechin is highly reactive and undergoes auto-oxidation to form reactive oxygen species, resulting in polymerization and decomposition (ISHII *et al.*, 2011). Nanocarriers are a promising option to overcome EGCG shortcomings for its application as a therapeutic agent.

Functionalization of TuMV derived VNPs with EGCG for antiviral applications

The Zika virus is a *Flavivirus* of the family *Flaviviridae* that is associated with neurological complications and severe malformations in humans (Musso and Gubler, 2016). This virus is classified as an arbovirus, which is an ecological term that defines viruses that are maintained by the horizontal transmission between vertebrate hosts and arthropods. The most common transmission method is through a bite from an infected *Aedes* species mosquito (Gutiérrez-Bugallo *et al.*, 2019). Although prior to 2007, reported transmissions were very low, an outbreak in the Federate States of Micronesia resulted in an estimated 5000 infections (Duffy *et al.*, 2009). Since then, other concerning outbreaks have occurred in French Polynesia (32,000 people), New

Caledonia, Easter Island, Samoa, Thailand, Philippines, and Brazil, amongst others (Petersen *et al.*, 2016).

Amongst the other mentioned health-promoting activities of EGCG, antiviral activities have been reported. Previous reports have shown to inhibit the maturation, replication, and infectivity of various viruses such as zika virus (ZIKV), herpes simplex, and coronavirus (Clark *et al.*, 1998; Friedman, 2007). Even though exact mechanisms on how EGCG promotes antiviral activities are yet to be fully described, there are a number of documented activities such as its antioxidant capacity, its ability to suppress viral RNA synthesis, to disrupt cell membranes, to bind to virulent proteins, and to trigger host immunity response (Friedman, 2007). In specific, it has been found that EGCG inhibits the entrance of ZIKV by exerting direct action on the viral activity (Carneiro *et al.*, 2016).

2.2 Experimental section

Chemical conjugation of EGCG

Production and purification of TuMV VNPs. TuMV VNPs were obtained as previously described on Chapter 1, page 24.

Chemical conjugation of EGCG. EGCG was chemically bound to VNPs through a Mannich reaction. Previously, a buffer exchange was required to eliminate the amines present in TRIS buffer. VNPs were centrifuged (50 min, 80,000 x g, 4 °C) and resuspended in 10 mM HEPES, pH 7.5, to a final concentration of 2 mg/ml. EGCG

(Sigma-Aldrich, Germany) was dissolved in MES, pH 6.7, to a final concentration of 5 mg/ml. Different amounts of EGCG were added to 100 µg of VNPs and 5 µl of 37% formaldehyde, and the final volume was adjusted to 115 µl. The reaction was stirred and incubated at room temperature for 72 hours. EGCG excess was removed by centrifugation (50 min, 80,000 x g, 4 °C), the pellet washed twice and resuspended in 10 mM HEPES, pH 7.5, to a final concentration of 1 mg/ml of VNPs.

EGCG coupling estimation. The amount of EGCG bound to TuMV was analyzed by modifying a previously described method for the determination of tannin-protein complexes (Makkar, Dawra and Singh, 1988; Makkar, 2000). To microwells from a 96-well plate, 15 µl portions of EGCG-VNPs solution were added and incubated with 15 µl of 10mM HEPES pH 7.5, 90 µl of SDS-triethanolamine solution (SDS 1% (w/v), triethanolamine 7% (v/v)), and 30 µl of a ferric chloride reagent (0.01 M ferric chloride in 0.1 M HCl) for 30 minutes, after which absorbance at 510 nm was recorded. A calibration curve was constructed by plotting absorbances for different, known amounts of EGCG.

Electron microscopy. Electron microscopy grids (400-mesh nickel, carbon-coated) were prepared by gently placing them, film-down, on top of a 10 µl sample drop and incubated for 15 minutes at room temperature. Next, it was washed three times (three minutes each) with distilled water. The grid was stained with 2% (w/v) uranyl acetate for 2 min. Samples were examined on a transmission electron microscope (JEM JEOL 1010, Japan).

Functionalization of TuMV derived VNPs with EGCG for antimicrobial applications

Assay for antimicrobial activity of TuMV-EGCG VNPs. The antimicrobial activity of EGCG-VNPs was evaluated against *Dickeya dadantii*, *Sarcina lutea*, and *Pseudomonas aeruginosa* by disk susceptibility tests. Overnight bacterial cultures were grown in Mueller-Hinton media (MH), adjusted to an OD₆₀₀ of 0.1, and inoculated on Muller-Hinton agar (MHA) plates. Antibiotic disks containing 10 µg of EGCG-VNPs or 5 µg of kanamycin (as control) were placed on the plates and incubated for 24 hours at 37 °C. To evaluate the ability of EGCG-VNPs to inhibit bacterial growth in liquid media, overnight cultures were grown on MH (for *S. lutea*), or minimal M9 medium containing 48 mM Na₂HPO₄, 22 mM KH₂PO₄, 9 mM NaCl, 19 mM NH₄Cl, pH 7.0 supplemented with 2 mM MgSO₄, 100 µM CaCl₂, and 20 mM glucose (for *D. dadantii* and *P. aeruginosa*). The overnight cultures were adjusted to OD₆₀₀ of 0.1, and 150 µl portions were placed in microwells from a 96-well-plate. Different amounts of EGCG-VNPs and free EGCG were added. The plates were incubated in a shaker (200 rpm) for 24 hours at 37 °C. After incubation, absorbance was recorded at 600 nm, and a single plate-serial dilution spotting (SP-SDS) was performed (Thomas *et al.*, 2015). The results of three plates with three replicates corresponding to a particular sample were averaged, and this value was regarded as the minimal inhibitory concentration (MIC) of EGCG -VNPs against each microorganism.

Biofilm formation assays. For the evaluation of the inhibition of biofilm formation by *P. aeruginosa*, the OD₆₀₀ of an overnight culture was adjusted to 0.1 with minimal Mg medium. 150 µl portions were placed in microwells from a 96-well plate. Plates were incubated in a shaker (200 rpm) for 24 hours at 37 °C. After incubation, microwells were carefully washed three times in order to remove planktonic cells. 180 µl Crystal Violet 2% (w/v) in methanol was added and incubated at room temperature for 10 minutes. Wells were washed three times to remove dye excess, 180 µl ethanol was added, and absorbance was recorded at 550 nm.

Evaluation of EGCG-TuMV infectivity in *Chenopodium quinoa*. For the assessment of the infectivity of EGCG-TuMV, groups of six three-week-old plants of *Chenopodium quinoa* were inoculated with different solutions: HEPES 10 mM pH 7.2, TuMV (isolate UK 1, 1 mg/ml in HEPES pH 7.2), EGCG (1 mg/ml in HEPES pH 7.2) and EGCG-TuMV (1 mg/ml in HEPES pH 7.2). Plants were dusted with carborundum, and two leaves per plant were manually inoculated with 5 µl of the corresponding solution. Plants were observed for ten days, symptoms were recorded, and two 1-cm leaf disks per leaf were collected and homogenized in 500 µl of extraction buffer (Na₂CO₃ 50 mM pH 9.6 PVP 40 1% (p/v)).

In order to determine the presence of TuMV, an indirect ELISA assay was performed. 100 µl of each homogenized sample was placed on a 96-well plate and incubated overnight at 4 °C. Then, microwells were washed by three 5-minute incubations with PBS-Tween20 0.05% (v/v). Anti-poty primary antibody (Agdia, United States) diluted

1:500 in antibody solution (Na_2HPO_4 10 mM, NaCl 150 mM, PVP40 1%(p/v), Tween20 0.05% (v/v), BSA 2% (p/v)) was added and incubated at room temperature for one hour. Microwells were washed by three 5-minute incubations with PBS-Tween20 0.05% (v/v). Alkaline phosphatase anti-mouse antibody (Agdia, United States) diluted 1:30000 in antibody buffer was added and incubated for two hours at room temperature. Microwells were washed by three 5-minute incubations with PBS-Tween20 0.05% (v/v). Finally, alkaline phosphatase substrate was added, and absorbance was recorded at 405 nm.

Statistical analysis of the data was performed using Prism 9, a one-way ANOVA test followed by Bonferroni's multiple comparison test.

Antimicrobial activity against *P. syringae*. The antimicrobial activity of EGCG-VNPs against *Pseudomonas syringae* pv. strain DC3000 (*PsPto*) was tested in liquid media. Overnight cultures were grown on King's B (KB) medium. The overnight cultures were adjusted to OD_{600} of 0.1, and 150 μl portions were placed in microwells from a 96-well-plate. Different amounts of EGCG-VNPs and free EGCG were added. The plates were incubated in a shaker (200 rpm) for 24 hours at 28 °C. Absorbance (OD_{600}) was recorded.

Tomato virulence assays. *PsPto* was grown at 28 °C for 24 hours on King's B (KB) medium under dark conditions. A cell suspension was prepared as previously described (Santamaría-Hernando *et al.*, 2018). Cells were resuspended in 10 mM

MgCl₂ and adjusted to a final concentration of 10⁸ CFU/ml. Silwet-77 was added to the bacterial suspension to a final concentration of 0.02% (v/v) before inoculation.

As depicted in Figure 2.4, a group of 3-week old tomato plants (*Solanum lycopersicum* cv Moneymaker) was pretreated with EGCG-VNPs. A solution of EGCG-VNPs in 10 mM HEPES, pH 7.5, to a final concentration of 50 µg/ml was prepared. 25 µl of EGCG-VNPs solution was carefully spread on the surface of 4 leaves of each plant (both sides). Plants were let to dry for 1 hour. Then, all plants were inoculated with DC3000 by spraying six leaves of each plant (6 plants per group) with cell solution. Plants were let to dry for two hours. After the drying time elapsed, a second group of plants was treated with the EGCG-VNPs solution. Plants were incubated in a growth chamber at 25 °C and 60% RH with a daily light period (100 µE/m²s) of 12 h. After 72 hours, a third group of inoculated plants was treated with the EGCG-VNPs solution. Six days after inoculation, leaf symptoms were recorded, and five 1-cm leaf disks per plant were sampled. To eliminate surface bacteria, the infected leaf disks were washed twice with 10 mM MgCl₂. After washing, leaf disks were homogenized in 10 mM MgCl and drop plated on KB agar. Plates were incubated at 28 °C for 48 hours, the average number of bacteria per cm² of each sample was determined and the data sets were analyzed using Minitab using GzLM, followed by the Least Significance (LSD) test.

Figure 2. 4 | Graphic representation of virulence assay. (A) Control group. (B) Pretreatment group: Leaves were coated with EGCG-TuMV before inoculation. (C) Leaves were treated with EGCG-TuMV three hours after inoculation. (D) Leaves were treated with EGCG-VLPs 72 after inoculation. Plants were analyzed six days after inoculation.

Functionalization of TuMV derived VNPs with EGCG for antitumor applications

Antitumor *in-vitro* assay. 8000 Caco-2 (Colorectal adenocarcinoma, ATCC® HTB-37™), A459 (lung carcinoma, ATCC® CCL-185™), MCF7 (mammary gland, ductal carcinoma, ATCC® HTB-22™), and MCF10A (mammary gland, ATCC® CRL-10317™) cells were cultured in plated in a 96-well microtiter plate and allowed to attach overnight in growth medium. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin was used for cell culture of Caco-2, A459, and MCF7. DMEM-F12 supplemented with 5% FBS, 20 ng/ml epidermal growth factor (Agrenvec, Spain), 10 µg/ml insulin (Sigma-Aldrich,

Germany), 0.5 µg/ml dehydrocortisone (Sigma-Aldrich, Germany), 100 ng/ml cholera toxin (Sigma-Aldrich, Germany), and 1% (v/v) penicillin-streptomycin was used for cell culture of MCF10A. After overnight incubation, the medium was aspirated, and each well was washed three times with PBS. Medium with EGCG, EGCG-TuMV, TuMV, or no supplement was added, and cells incubated for 24 hours. For cell proliferation, MTS assay CellTiter 96®One solution (Promega, United States) was performed according to the instructions from the manufacturer. After 24-hour incubation, the medium was aspirated, wells were washed three times with PBS. 100 µl serum-free medium and 20 µl of CellTiter solution were added. Cells were incubated at 37 °C, humidified 5% CO₂ atmosphere. Absorbance was recorded at 490 nm.

Apoptosis/Necrosis *in-vitro* assay. 200000 MCF7 cells were cultured in plated in a 6-well microtiter plate and allowed to attach overnight in growth medium. Cells to be analyzed by confocal microscopy were grown over glass covers. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin was used for cell culture. After overnight incubation, the medium was aspirated, and each well was washed three times with PBS. Medium with EGCG (120 µM), EGCG-TuMV (120 µM), TuMV (0.05 µg/µl), or no supplement was added, and cells incubated for 24 hours. After treatment, incubation cells were washed three times with PBS. Apoptosis and necrosis were recorded using the Apoptosis/Necrosis Assay Kit (Abcam, United States). Cells to be analyzed by cytometry were collected, cells that were analyzed by microscope were handled in glass covers. Apopxin green indicator, 7-AAD necrotic cells indicator, and CytoCalcein

were added to the cells. Cells were incubated for 30 minutes at room temperature. Cells were washed three times with PBS. Cytometry assays were performed on the AccuriTM C6 cytometer. Cells to be observed on a confocal fluorescence microscope (Zeiss LSM 880) were not fixed.

Functionalization of TuMV derived VNPs with EGCG for antiviral applications

Virucidal effect assay. Vero cells (*Cercopithecus aethiops*, kidney, ATCC® CC-81) were grown at 37°C in 5% CO₂ atmosphere in DMEM supplemented with 2 mM glutamine, penicillin-streptomycin, and 5% FBS. Viral strain ZIKV PA259659 (ZIKV) was used in these tests (Martin-Acebes *et al.*, 2014). To test virucidal effects, ZIKV was incubated with different concentrations (0-100 µM) of EGCG or EGCG-TuMV for 1 hour at 37 °C. 200000 Vero cells were seeded in 12-well plates. Then cells were incubated with corresponding treated or untreated virus at 37°C. Cell infection was carried out with viruses at a multiplicity of 1 plaque-forming unit (PFU)/cell. After the time elapsed, the inoculum was removed, and cell incubation continued in culture medium with 2% FBS and 1% low-melting-point agarose. Viral titers were determined by plaque assay in semisolid agarose medium at 72 h after infection. The multiplicity of infection (MOI) used in each experiment was expressed as PFU/cell. Plaques were visualized after formaldehyde 4% fixation and crystal violet staining (0.3% crystal violet in 2% formaldehyde and 10% ethanol).

Inhibition of infection effect assay. First, 25000 Vero cells were seeded in 96-well plates and incubated at 37°C in 5% CO₂ for 24 hours. Treatment-growth medium with different concentrations (0-100 µM) with EGCG, EGCG-TuMV, and TuMV were prepared. Growth medium was removed, cells were gently washed with PBS, and the treatment-growth medium was added. Then, cell infection was carried out with viruses at a multiplicity of 1 plaque-forming unit (PFU)/cell. After 24 hours of incubation, cell viability was measured, and the medium from each treatment was recovered. Cell viability was determined by measuring the extracellular ATP content with CellTiter-Glo luminescent cell viability assay (Promega, United States). Virus extracellular production was tested by using the recovered treatment mediums to infect priorly seeded 12-well plates. Viral titers were determined as previously described in the virucidal effect assays.

2.3 Results and discussion

Chemical conjugation of EGCG

As previously mentioned, the transfer towards specific applications of EGCG has proven limited due to its stability. The incorporation of EGCG into nanoparticles is a promising strategy to help the bioavailability and, as a result, opens the path for the exploitation of its many beneficial activities. Protein-based NPs are a highly favorable alternative since, in comparison to metallic-based NPs, lack such environmental disadvantages. Also, their highly modifiable surface allows a high versatility on the design of the VNPs. The symmetry of TuMV makes it an attractive candidate to be used as a nanocarrier. Lysine residues from the TuMV capsid protein are mainly exposed on the surface and can be used to functionalize the virion (Yuste-Calvo *et al.*, 2019).

Attachment of EGCG molecules to TuMV-derived VNPs can be achieved through lysine conjugation. EGCG molecules bound to VNPs will be likewise exposed to the outside environment (Figure 2.5). EGCG binds to the external surface of cell membranes, thus damaging or fragmenting the lipid bilayer (Das *et al.*, 2014). The latter suggests that the exposure of EGCG to the outside environment is important since this configuration would allow for a high number of available EGCG molecules concentrated within a relatively small area (high effective concentration). In order to obtain such EGCG-TuMV nanoparticles, a Mannich condensation was used to target both the polyphenolic compound and primary amines from lysine residues, as shown in Figure 2.6.

Figure 2. 5 | Schematic representation of EGCG-TuMV nanoparticle synthesis. The step of discarding the excess of reagents not incorporated into particles is not represented.

Figure 2. 6 | Mannich condensation between EGCG and TuMV.

Optimization of the ratio of EGCG to VNP in the reaction was carried out by testing different concentrations of EGCG. After reacting, unbound EGCG was removed by ultracentrifugation, and the resulting conjugated EGCG was estimated. TuMV concentration was kept constant at 0.87 $\mu\text{g}/\mu\text{L}$. As depicted in Figure 2.7, an EGCG concentration of 2.27 mM in the reaction yielded a high amount of conjugated EGCG. One higher initial concentration of initial EGCG in the series was tested (2.66 mM), but this was too close to concentrations resulting in a high degree of protein precipitation (results not shown), a situation to be expected since aggregation from the binding at high EGCG loading capacities has been described (Xue *et al.*, 2014). Thus, we decided to work at 2.27 mM. At this chosen working conditions, the

estimated amount of EGCG coupled to TuMV was $1.1 \pm 0.1 \mu\text{g EGCG} / \mu\text{g TuMV}$. These conjugated VNPs were used in subsequent biological assays.

Figure 2.7 | EGCG-TuMV characterization. (a) Amount (μg) of EGCG conjugated to TuMV as a function of EGCG concentration in the initial condensation reaction. TuMV concentration was maintained constant in the reaction at 0.87 mg/ml . (b) Electron microscopy (TEM) micrographs of purified TuMV (i) or EGCG-TuMV particles (ii).

Although EGCG is known to interact non-covalently with proteins (Wang *et al.*, 2014), as seen in Figure 2.8, those VNPs that were produced using formaldehyde yielded a higher amount of linked EGCG. Since polyphenols are known to be highly reactive with proteins and tend to aggregate (Hagerman, 2012), an important consideration was the maintenance of TuMV particle structure. TEM images, shown in Figure 2.7, did not reveal significant changes in the VNP structure after EGCG

conjugation. The characteristic, filamentous shape of TuMV particles remained mostly unmodified.

Figure 2. 8 | Amount (mg) of EGCG conjugated per mg TuMV as a function of the presence of formaldehyde. TuMV concentration was maintained constant in the reaction at 0.87 mg/ml. Data represent the means and standard errors of three replicates ($p < 0.05$, One-way ANOVA).

Antimicrobial activity of EGCG-TuMV VNPs

Once EGCG-TuMV conjugation was completed, the maintenance of EGCG-derived antimicrobial activity was tested. First, a disk diffusion susceptibility test was performed on MHA plates. As shown in Figure 2.9, there was a good antimicrobial activity for both *Dickeya dadantii* and *Sarcina lutea*. In both cases, we found inhibition around disks containing 10 µg of EGCG-TuMV.

Figure 2. 9 | Inhibition of bacterial growth by EGCG-TuMV in solid media plates. Inhibition haloes were obtained for both *D. dadantii* and *S. lutea*. The inoculum was adjusted to an OD₆₀₀ 0.1 and cultured overnight on HMA. Kanamycin was used as a positive inhibition control.

In order to establish the limits of the antimicrobial activities, tests in liquid media were performed. It has been previously reported that EGCG shows lower antimicrobial activity against Gram-negative bacteria due to their outer layer of liposaccharides (Jeon *et al.*, 2014). *D. dadantii* and *Pseudomonas aeruginosa* (Gram-negative) and *S. lutea* (Gram-positive) were compared. Experiments were repeated three times in order to ensure reproducibility with three replicates each. Cultures were performed in 96-well microtiter plates with minimal M9 medium supplemented with glucose for *D. dadantii* and *P. aeruginosa* and MH medium for *S. lutea*. For all

strains tested, as shown in Figure 2.10, the absorbance of overnight cultures showed the same behavior. Culture growth was notably lower in the cases of higher concentration of EGCG-TuMV nanoparticles, suggesting that the antimicrobial effect of the nanoparticles is concentration-dependent. The observed antimicrobial effect of the EGCG-TuMV nanoparticles can be attributed to the conjugation of the polyphenolic molecule, given that cultures exposed to purified TuMV did not show growth inhibition (Figure 2.10). A difference in the antimicrobial activity of free EGCG vs. EGCG-TuMV particles was observed, with free EGCG moderately inhibiting tested cultures.

Figure 2. 10 | Inhibition of bacterial growth by EGCG-TuMV in liquid cultures. Graphs represent the absorbance (OD 600 nm) of *P. aeruginosa*, *D. dadantii*, or *S. lutea* cultures after exposure o/n to increasing concentrations of free EGCG, TuMV, or EGCG-TuMV. Data represent the means and standard errors of three replicates (p < 0.05, Two-way ANOVA).

The minimum inhibitory concentrations (MICs) shown in Figure 2.11 were established as the minimum concentration at which 99.9% of bacterial growth was inhibited. First, cultures were performed in a 96-well microtiter plate with minimal M9 or MH media and incubated overnight at 37 °C. After incubation, a single plate-

serial dilution spotting (SP-SDS) was performed. The MICs found for *D. dadantii*, *P. aeruginosa*, and *S. lutea* were 16.7, 50.0, and 24.8 $\mu\text{g/mL}$, respectively. Direct inoculation from these inhibited cultures did not yield any colonies, indicating that EGCG-TuMV nanoparticles are bactericidal, an observation that correlates with prior studies on the antimicrobial effects of free EGCG (Ikigai *et al.*, 1993).

Figure 2. 11 | Quantification of inhibition through colony forming unit (CFU) counting after bacterial growth in liquid media. CFU count of *P. aeruginosa* and *D. dadantii* after overnight incubation a 37 °C. The results for *S. lutea* are shown in a numerical form, because in most cases the results were too high to count (THTC). Data represent the means and standard errors of three replicates ($p < 0.05$, Two-way ANOVA).

MIC determinations confirmed that the inhibitory effects of EGCG-TuMV particles were higher than those of free EGCG for all bacteria tested. In the case of *P. aeruginosa*, a MIC of 400 $\mu\text{g/mL}$ has been reported for extracted EGCG (Jeon *et al.*, 2014), which is still much higher than 50 $\mu\text{g/ml}$ we found for EGCG-TuMV.

Contrary to previous reports on a higher susceptibility to EGCG by Gram-positive bacteria, we found that *D. dadantii* (Gram-negative) had a lower MIC in comparison to *S. lutea* (Gram-positive). Given that similar results have also been found in other

studies (Sun, Zhang and Li, 2011) it appears that generalizations regarding differential sensitivity to EGCG by Gram-positive and Gram-negative bacteria are still premature. Within the concentration range tested, free EGCG did not show complete inhibition of culture growth for any of the bacteria tested. Since the same batch of purified EGCG was used both for these tests and for nanoparticle synthesis, other factors that affect free EGCG stability, such as pH, exposure to light, and susceptibility to redox reactions, could be playing a role. It has been reported that coupling EGCG to protein stabilizes the polyphenolic compound under physiologic conditions (Li *et al.*, 2014a). The latter, together with the exposed nature of EGCG molecules conjugated on the surface of the particle, might explain the difference in antimicrobial effectiveness observed in the present study. Finally, as seen in Figure 2.12, EGCG-VNP retained good antimicrobial activity after six months of being produced, stored at 4 °C in 10 mM HEPES, which is an indicative that EGCG conjugation with TuMV might increase the time in which EGCG can be used as an antimicrobial.

Figure 2. 12 | Inhibition of bacterial growth by EGCG-TuMV in liquid cultures. Graphs represent % of the absorbance (OD 600 nm) of Control *D. dadantii* cultures after exposure o/n to EGCG-TuMV that have been stored during increasing amounts of time.

Evaluation of biofilm formation

Bacterial biofilms are multicellular aggregates in which cells are embedded in an extracellular matrix, much more refractory to antimicrobials. This matrix encases bacterial cells and provides protection by performing different tasks. It confers structural stability and flexibility, traps antimicrobial compounds, and maintains homeostatic conditions within the biofilm (Flemming *et al.*, 2016). Because of this biofilm-conferred infection resilience, the need for new antimicrobial agents addressing this problem has become evident. Biofilm-associated antimicrobial resistance is not acquired by classical antibiotic resistance such as mutations in antibiotic targets but is part of regular bacterial regulatory repertoire (Hengge, 2019). Specifically, EGCG is an attractive candidate since EGCG-induced bacterial membrane

damage deters the ability of the bacteria to bind to each other to form biofilms (Blanco *et al.*, 2005).

EGCG-TuMV ability to inhibit biofilm formation was tested using cultures of *P. aeruginosa* in M9 supplemented with glucose exposed to different concentrations of EGCG and EGCG-TuMV and incubated for 24 hours, after which biofilm was quantitated with crystal violet. As shown in Figure 2.13, EGCG-TuMV VNPs were capable of inhibiting *P. aeruginosa* biofilm formation at a concentration of 42 µg/ml. Biofilm inhibition was dependent on EGCG-TuMV VNP concentration and was clearly different from the effects found with free EGCG and TuMV treatments.

EGCG has been reported to act against *P. aeruginosa* biofilms by interfering with the assembly of amyloid fibers and by impairing quorum sensing (Yang *et al.*, 2010; Stenvang *et al.*, 2016). These effects produce biofilms that are less stiff and more susceptible to antibiotics. On the other hand, it has also been reported that EGCG interferes with *P. aeruginosa* swarming motility and enhances biofilm formation (O'May *et al.*, 2012). The latter was also found in our tests. Free EGCG enhanced biofilm formation in a concentration-dependent manner (Figure 2.13). However, nanoparticles loaded with EGCG clearly inhibited the formation of biofilm of *P. aeruginosa*.

Figure 2.13 | Biofilm adhesion assay of *P. aeruginosa*. Cultures were grown for 24 hours at 36°C in 96-well microtiter plates containing M9 media. Biofilm formation, indicated by crystal violet staining, was measured at 550 nm. Data represent the means and standard errors of three replicates.

Evaluation of EGCG-TuMV for plant protection

TuMV-EGCG infectivity

In order to assess the potential application of EGCG-TuMV in plant protection, infectivity in *Chenopodium quinoa* was evaluated. *Chenopodium quinoa* is a useful model for TuMV infectivity since symptoms of TuMV include lesions that work as visual cues (Figure 2.14A). None of the EGCG-TuMV inoculated plants showed lesions (Figure 2.14A). An ELISA was performed to detect the presence of TuMV in plant extracts. Extracts from plants that were inoculated with TuMV were positive while the virus was not detected on plants inoculated with the conjugated nanoparticles, which further demonstrate that TuMV, when bound to EGCG, is not capable of infecting plants (Figure 2.14B).

The latter was expected since the modification of the overall net charge of TuMV has an effect on its infectivity (Revers and García, 2015a). Nanoparticles coated with EGCG tend to have a negatively charged surface at pH 7.0 (Li *et al.*, 2014b; Suresh *et al.*, 2015). Furthermore, TuMV is produced using a clone that is non-aphid transmissible and cannot be transmitted through seeds or contact between plants (Tourinho *et al.*, 2008). The lack of infectivity, in combination with the limited transmission of TuMV, suggest an increased biosafety which also show the potential EGCG-TuMV nanoparticles in plant protection.

Figure 2. 14 | (A) Infection symptoms of TuMV (i), while no symptoms were observed on plants treated with EGCG-TuMV (ii). (B) ELISA assay between plant samples. Data represent means and standard errors of an assay with twelve replicates ($p < 0.0001$, $*p < 0.0001$, One-way ANOVA followed by Bonferroni's multiple comparison test).

Evaluation of virulence in tomato plants

In order to explore the potential of EGCG-TuMV VNPs as an antimicrobial agent for plant protection, *Pseudomonas syringae* was tested in tomato plants (*Solanum lycopersicum* cv MoneyMaker). *P. syringae* is a bacterial plant pathogen of significance since it causes significant losses in agronomically relevant crops. Throughout the years, *P. syringae* has been responsible for recurring chronic diseases in a wide variety of crops, making it a problematic pathogen (Hulin et al., 2018). This phytopathogen is capable of bacterial virulence, which means that it is capable of suppressing host immunity and cause disease (Nomura et al., 2006). An antimicrobial assay in liquid media was performed (Figure 2.15) to assess antimicrobial activity against *PsPto*. The inhibitory concentration of EGCG-TuMV was found at 33.33 µg/ml. As with the other tested strains, the inhibitory concentration was lower than the reported at 1 mg/ml from prior studies (Lovato et al., 2019).

Figure 2. 15 | Inhibition of bacterial growth by EGCG-TuMV in liquid cultures. The graph represents the absorbance (OD 600 nm) of *Pseudomonas syringae* pv. *Tomato* strain DC3000 (PsPto) cultures after overnight exposure to increasing concentrations of free EGCG, TuMV, or EGCG-TuMV. Data represent the means and standard errors of three replicates ($p < 0.05$, Two-way ANOVA).

In vivo assays were carried out by testing EGCG-TuMV treatments at different inoculation times (Figure 2.4). Since the behavior of EGCG-TuMV nanoparticles over the surface of a plant is unknown, we opted to test applications at different inoculation times. As shown in Figure 2.16A, treatments after bacterial inoculation resulted in a negative effect on bacterial virulence. The Control group behaved as expected, and observable disease symptoms (Figure 12.16B) also correlate with the aforementioned results since treated samples showed lower signs of PsPto infection. These are promising results since it has been reported that *in vivo* conditions may vary from *in vitro* results; in particular, it has been reported EGCG extracts yield moderate to no inhibition *in vivo* assays (Lovato *et al.*, 2019; Yang and Zhang, 2019).

Figure 2. 16 | PsTo virulence is affected by EGCG-TuMV treatment. (A) Virulence of PsPto, as assessed on bacterial population sizes in tomato leaves at six days after inoculation. Data represent the means and standard errors of three replicates; groups that do not share a letter are significantly different from each other ($p < 0.05$, GzLM followed by LSD test). (B) Disease symptoms on leaves from each treatment (i) Control group, (ii) EGCG-treatment prior inoculation, (iii) EGCG-TuMV treatment 3 hours after inoculation, and (iv) EGCG treatment 72 hours after inoculation.

Obtained data suggest that the time of treatment has an effect on EGCG-TuMV effectivity as antimicrobial. In particular, treatment prior to inoculation yielded higher bacterial virulence. There are a number of possible reasons for these results. First, it has been reported that concentrations lower to MICs may increase biofilm formation (O'May *et al.*, 2012). Also, it has been found that EGCG can enhance biofilm formation of *P. syringae* strains at a concentration of 10 $\mu\text{g/ml}$ (Tomihama, Nishi and

Arai, 2007). Furthermore, the method of EGCG-TuMV application may have increased infectivity since it was mechanical. This can be an issue since *P. syringae* lives on the plant surface as an epiphyte and enters the plant through natural openings and wounds (Xin, Kvitko and He, 2018). The mechanical application of EGCG-TuMV may have increased the number of superficial wounds that *P. syringae* can use for plant infection. EGCG treatment before inoculation (Fukai, Ishigami and Hara, 1991; Lovato *et al.*, 2019) has been studied with adverse effects on plant virulence, which suggests that it is possible to approach treatment at this time. Further studies have to be done to determine if EGCG-TuMV treatment prior inoculation can be achieved by other application techniques without increasing virulence.

On the other hand, the exposure to EGCG-TuMV after inoculation produced lower microbial infection. Although EGCG antimicrobial mechanisms are yet to be fully explained, it has been reported that it stops bacterial growth and hinders *P. syringae* swimming motility (Lovato *et al.*, 2019; Yang and Zhang, 2019). Both factors are crucial for epiphyte survival and directly affect bacterial virulence (Xin, Kvitko and He, 2018). Tests results indicate activity over *P. syringae* up to three days after inoculation.

Each side of a treated sample was coated with 25 µl of EGCG-TuMV solution (50 µl total) at a concentration of 50 µg/ml. The latter amount is lower than those reported in the literature in which 4.7 mg/mL EGCG is used with moderate effect (Lovato *et al.*, 2019). Although it is yet not possible to determine if EGCG-TuMV is capable of yielding on complete inhibition of treated plants, these results suggest that EGCG-TuMV is capable of hindering *PsPto* virulence while being exposed to *in*

vivo conditions. This is of particular importance since EGCG high reactivity and ease for oxidation due to oxygen, light, and water exposure, limit its applications (Mereles and Hunstein, 2011; Potenza *et al.*, 2020). The aforementioned reasons make necessary the use of alternative approaches. The multimeric presentation of EGCG in the nanoparticle's surface presumably increases its potential as a tool for microbial control.

Antitumor activity of EGCG-TuMV VNPs

EGCG has proven to have several effects on tumor cells and tumor stages. For this reason, the focus on this polyphenol has increased over the years. Proliferation assays performed on A459 (lung carcinoma), MCF7 (ductal carcinoma from mammary gland), and Caco-2 (colon adenocarcinoma) showed that EGCG-TuMV not only retained dose-dependent inhibition activities but also increased its effects on tumor cell lines in comparison to free EGCG (Figure 2.17). Inhibition of cell proliferation is consistent with previous reports for all tumor cell lines (Relat *et al.*, 2012; Zhang *et al.*, 2012; Ranzato *et al.*, 2014). Also, EGCG effects over MCF10A (nontumorigenic mammary gland) are dose-dependent, whereas cytotoxicity was observed at the highest tested concentration.

Similar to obtained results in the antimicrobial assays, free EGCG results differ from those previously reported. IC₅₀ has been found at 19-50 μ M for MCF7 (Zhang *et al.*, 2009; Ranzato *et al.*, 2014), 50->500 μ M for CaCo-2 (Zhang *et al.*, 2012; Song *et al.*, 2014; Silva *et al.*, 2019), and 80-265 μ M for A459 (Relat *et al.*, 2012; Jeon *et al.*, 2014). While it is not possible to calculate IC₅₀ values with the presented data, tested

concentrations of free EGCG were higher than IC₅₀ values reported for MCF7 and CaCo-2. Also, for MCF10A, cytotoxic effects are reported starting at a concentration of 87 μ M (Rathore *et al.*, 2012). The variance of all reported IC₅₀ values for each cell line also reflects how results may be different in different laboratories. There is a number of factors to consider in order to understand the possible causation for this variability in results. The exact mechanism of EGCG over cells is yet to be understood. Also, EGCG is a reactive molecule prone to oxidation when exposed to water, light, and oxygen. The presentation of this molecule in a nanoparticle may present a possible avenue to increase its stability and diminish the variability of the degree of its activity.

Figure 2.17 | Proliferation assays of tumor cell lines A459 (lung carcinoma), MCF7 (ductal carcinoma from mammary gland), CaCo-2 (colon adenocarcinoma), and non-tumor cell line MCF10A (mammary gland). Cells were exposed to different concentrations of EGCG, EGCG-TuMV, and TuMV for 24 hours. Percentages are calculated in relation to their respective untreated control. Data represent the means and standard errors of three replicates ($p < 0.05$, Two-way ANOVA).

In all tested concentrations of EGCG-TuMV, a cytotoxic effect was observed for MCF7 while only the higher tested concentration showed cytotoxic effects in MCF10A non tumor cell line (Figure 2.18). This is consistent with previously reported data since it has been found that EGCG, compared with traditional cancer treatment molecules, appears to address specific targets unique to cancer cells while not harming normal cells (Wu, 1992; Babich *et al.*, 2005; Singh, Shankar and Srivastava, 2011). This highlights the potential of EGCG-TuMV as a possible treatment for cancer.

Figure 2. 18 | Proliferation comparison between MCF10A and MCF7 after different concentrations (196.3, 130.9 and 65.4 (M) of EGCG-TuMV ($p < 0.05$, $*p < 0.0001$, Two-way ANOVA test followed by Šídák's multiple comparisons test). Percentages are calculated in relation to their respective untreated control.

In order to gain further insight into the possible effects of EGCG-TuMV over cells, an apoptosis/necrosis assay was performed with MCF7 cells. Apoptosis can be

induced via intrinsic and extrinsic pathways. The intrinsic pathway can be induced through intracellular stresses (DNA damage or oxidative stress), while the extrinsic pathway is induced by ligands and necrosis factors (Rady *et al.*, 2018). It has been observed that EGCG may induce apoptosis through both pathways (Shankar, Suthakar and Srivastava, 2007; Md Nesran *et al.*, 2019). As seen in Figure 2.19A, samples treated with EGCG-TuMV showed higher signs of necrosis in comparison to samples treated with free EGCG. This was further assessed by flow cytometry. Results obtained from flow cytometry analysis are summarized in Figure 2.19B. Based on the Apopxin/7-AAD stain, 97% of control group cells were found alive with values of 1%, 0.8%, 1.2% of necrosis, late apoptosis, and apoptosis accordingly. TuMV treated cells presented the same behavior. Within the group of cells that were treated with free EGCG, the number of alive cells diminished to 71.4% with values of 5.2% (necrosis), 11.4% (late apoptosis), and 12.0 % (apoptosis). Finally, within the group of cells that was treated with EGCG-TuMV, alive cells decreased to 21.4% while necrotic cells increased to 60.1%, late apoptosis increased to 15.0%, and apoptosis decreased to 3.5%. These results suggest that EGCG-TuMV increases the process of cell death, in specific necrosis, in MC7 cells.

Figure 2. 19 | Apoptosis/Necrosis assays. (A) MCF7 cells are exposed to EGCG-TuMV (120 μ M) or EGCG. Alive cells were stained with CytoCalcein Violet 450 (blue). Apoptotic cells were stained with apopxin green indicator (green). Necrotic cells were stained with 7-aminoactinomycin D (7-AAD, red). (B) Apopxin and 7-AAD assay of MCF7 cells when exposed to EGCG-TuMV (120 μ M), EGCG (120 μ M), and TuMV (0.05 μ g/ μ l) following a 24-hour treatment.

EGCG-TuMV antiproliferation activities, in combination to the increased cell death rate and differential activities amongst tumor and non-tumor cell lines, make it a potential strategy to circumvent EGCG natural limitations. Present *in vitro* studies suggest that the multimeric presentation of this molecule not only preserves its natural activities, but also enhances them.

Antiviral activity of EGCG-TuMV VNPs

As seen in Figure 2.20, free EGCG has a virucidal effect against ZIKV starting at 10 μ M while EGCG-TuMV diminished viral titer only at 100 μ M. TuMV does not have an effect on viral titer. These results suggest that EGCG acts directly on ZIKV, hindering its infection capacity. Also, while these results suggest that EGCG-TuMV may retain virucidal activity, effectiveness is diminished in comparison to free EGCG.

Figure 2. 20 | Virucidal assay on Vero cells. ZIKV was treated prior to infection with different concentrations of EGCG or EGCG-TuMV (0-100 μ M). Untreated ZIKV and treatment with HEPES were used as control. Data represent the means and standard errors of a set with three replicates.

As seen in Figure 2.21A, cytotoxicity was found when cells were treated with free EGCG and EGCG-TuMV at 100 μ M. Free EGCG inhibited infection at 10 μ M, which is consistent with previously reported data (Vázquez-Calvo *et al.*, 2017). EGCG-TuMV showed mild inhibition effects at 10 μ M, although at 100 μ M there was an apparent decrease in viral titer, cell viability was very low, which means that lack of viral activity is due to some type of change of cell conditions.

These results suggest that the multimeric presentation of EGCG in TuMV does not enhance EGCG natural antiviral activity. EGCG inhibition of ZIKV is not believed to be related to blockage of cellular receptors (Carneiro *et al.*, 2016) and rather, its main activity happens in destabilizing viral particles (Calland *et al.*, 2012). EGCG is a hydrophilic molecule that restricts approach to the sites of action. Prior studies have shown that enhancing lipophilicity of EGCG improves radical scavenging capacity, which has been correlated with viral inhibitory effects (Ho *et al.*, 2009; Zhong, Ma and Shahidi, 2012). The multimeric presentation of EGCG may have an impact on its solubility in water, which also may change the interaction with ZIKV. Further studies have to be made in order to understand the physicochemical characteristics of EGCG-TuMV.

Also, EGCG reported mechanisms on viral infectivity varies amongst different viruses. In some studies, it has been shown that EGCG destroys virion structures of members of the *Herpesviridae* family (Lyu, Rhim and Park, 2005; Isaacs *et al.*, 2011). It has been found that EGCG may suppress viral replication by modulation of cellular redox milieu (Ho *et al.*, 2009). Furthermore, the effects of EGCG even change on members of the same family (Carneiro *et al.*, 2016). Although inhibition results for ZIKV showed limited potential for this nanoparticle as an antiviral agent, different results may be obtained when testing other viruses. In order to further assess the antiviral activities of EGCG-TuMV, different avenues can be taken; its hydrophilicity can be modified, and more virus strains can be tested.

Figure 2. 21 | Inhibition of infection in Vero cells. Cells were treated with EGCG and EGCG-TuMV at early infection. Untreated ZIKV and treatment with HEPES were used as control. (A) Cell viability of cultures treated with EGCG, EGCG-TuMV, and HEPES. (B) Viral extracellular production of cells treated with EGCG and EGCG-TuMV. Data represent the means and standard errors of a set with three replicates.

2.4 Conclusions and perspectives

The conjugation of EGCG to TuMV does not impair its ability to act as an antimicrobial and antitumor agent. Rather, results suggest that this nanoparticle presentation promotes the aforementioned activities.

A common subject while exploring the deployment of EGCG for diverse applications is the wide number of activities that EGCG has over cells and proteins. For microbiological purposes, it affects bacterial DNA (Das *et al.*, 2014), causes oxidative stress (Cui *et al.*, 2012), and damages the cell membrane, increasing its permeability (Papuc *et al.*, 2017). In cancer applications, EGCG has also shown antitumor activity at diverse processes such as angiogenesis, necrosis, motility, invasion, migration, and metastasis markers (Almatrood *et al.*, 2020). EGCG acts at different levels in the cell, and there is still a lot to be learned about the complete scope of its effects. EGCG antiviral activity is complex and depends on both, virus infection process and host cell response (Li *et al.*, 2020). This wide variety of mechanisms in which EGCG has an effect opens the door for a number of possible applications despite its innate limitations.

Although we were able to demonstrate that EGCG-TuMV nanoparticles have potential as an antimicrobial and antitumor agent, the actual mechanisms of their inhibitory action are yet to be characterized. Considering the length of TuMV nanoparticles, *ca.* 700 nm, EGCG-TuMV nanoparticles are more likely to interact outside the cell membrane. Further studies are required to understand whether fragments or complete EGCG-TuMV particles are capable of passing through the cell

membrane and of having an effect from within. The antimicrobial, antibiofilm, and antitumor properties of EGCG-TuMV were not only maintained but also enhanced. One of the major advantages of VNPs is the versatility they provide. This technological platform may allow a rapid response to the increasing list of drug-resistant microbes or circumvent the secondary effect of chemotherapy. In the antiviral front, promising activities of EGCG-TuMV are yet to be found. This outcome may change by changing EGCG-TuMV lipophilicity or by exploring other virus strains.

Another possible avenue to explore is multiple functionalizations since VNPs have a number of sites that can be modified, and also there is an array of preparation methods for genetic and chemical manipulations with high flexibility of applications (Alemzadeh *et al.*, 2018).

TuMV derived VNPs is a technological platform that could be used to address issues such as microbial resistance or cancer treatment in different approaches. An additional target molecule could be conjugated to the nanoparticle, which may increase activity specificity or stability. Specifically, TuMV has proven to have the potential for multiple functionalizations (Yuste-Calvo *et al.*, 2019). This versatility, in combination with the results obtained in this study, makes TuMV VNPs good candidates to explore different arrays of molecules to act synergistically with EGCG.

CHAPTER 3: BIONANOTECHNOLOGY FOR TISSUE
ENGINEERING: DEVELOPMENT OF GELATIN
METHACRYLOYL (GelMA) ENRICHED WITH VIRUS
NANOPARTICLES (VNPs)

CHAPTER 3: BIONANOTECHNOLOGY FOR TISSUE ENGINEERING: DEVELOPMENT OF GELATIN METHACRYLOYL (GelMA) ENRICHED WITH VIRUS NANOPARTICLES (VNPs)

3.1 Introduction

Tissue engineering is a field of increasing interest since it is necessary to address current limitations and shortcomings in various areas such as regenerative medicine, personalized diagnosis and therapies, drug development, and biomedical research.

Modern tissue engineering techniques heavily rely on hydrogels due to their resemblance to the extracellular matrix (ECM). Usually, to resemble tissue microenvironments, hydrogels need to be supplemented with chemical and physical cues. Different strategies have been implemented to engineer more suitable hydrogels for tissue engineering applications. The addition of micro and nanoparticles is an implemented strategy for the enhancement of mechanical properties (Jiang *et al.*, 2017; El-Sayed *et al.*, 2019), the addition of sites for cell

attachment (Liu and Chan-Park, 2010), or to serve as vehicles for chemical compounds (Jha *et al.*, 2015; Browne *et al.*, 2018).

The addition of nanoparticles in tissue engineering is an attractive strategy to mimic the tissue microenvironment. Often, the material of nanoparticles used in tissue engineering includes inert materials such as carbon, silica, or metallic nanoparticles (Hassan *et al.*, 2019). The application of protein-based nanoparticles and, in particular, VLPs have been explored in the past (Dickmeis *et al.*, 2015), there are few examples, and their use has been limited. Viruses have been reengineered as functional scaffolds for cell adhesion, proliferation, and directing cell orientation (Zhao, Lin and Wang, 2015).

There are many advantages in the application of VLPs for tissue engineering. Viral capsids are made of protein; therefore, they are biodegradable, amenable to functionalization strategies that are more biocompatible (Bahadar *et al.*, 2016). Also, viruses are monodispersed assemblies with a well-organized structure that can be modified or assembled as scaffolds for tissue engineering (Dickmeis *et al.*, 2015). Plant-based VLPs offer an additional advantage since they cannot infect mammals relevant to biomedical applications.

As shown in Figure 3.1, TuMV has a suitable architecture for tissue engineering. TuMV particles are long (700 nm in length), thin (15 nm of diameter), flexuous, and modifiable through simple chemistries (Yuste-Calvo *et al.*, 2019). As shown previously, TuMV can be used to develop functional nanomeshes and presentation of antigens (Sánchez *et al.*, 2013; Cuenca *et al.*, 2016; González-Gamboa *et al.*, 2017). It

can also be modified to create a multimeric display of molecules of interest (Velázquez-Lam, Imperial and Ponz, 2020).

Figure 3.1 | General characteristics of TuMV.

Here, we use TuMV as an additive to engineering gelatin methacryloyl (GelMA) hydrogels for enhanced cell attachment, proliferation, and alignment. Furthermore, TuMV-GelMA hydrogels are amenable to different biofabrication strategies, including hydrogel casting and bioprinting.

3.2 Experimental Section

Production and purification of TuMV VNPs. TuMV VNPs were obtained as previously described on Chapter 1, page 24.

Atomic Force Microscopy (AFM). Fresh cleaved silica (DoñaChip, Mexico) was cut into squares of $\sim 1.5 \text{ cm}^2$ (silica holders). The silica holders were washed with 96% ethanol before use, and the surface was detached with tape. Each holder was coated with 5 μL of 0.02 mg/ml TuMV solution diluted in water and left to dry overnight. The immobilized TuMV was characterized using semi-contact mode AFM using silicon

AFM Probes (Tap190Al-G[®], USA) with a resonance frequency of 190 kHz, spring constant of 48 Nm⁻¹, and a scan rate of 1.0 Hz.

Transmission Electron Microscopy (TEM). TuMV nanoparticles were examined as described in Chapter 1, page 25.

Conjugation of VNPs and Alexa[™] Fluor[™] 555. TuMV-Alexa₅₅₅ were prepared as described in Chapter 1, page 27.

Conjugation of VNPs and EGFR. TuMV suspension buffer was changed for HEPES 20 mM pH 7.5 by ultracentrifugation (50 min at 50 000 x g), the pellet was resuspended up to a concentration of 1 mg/ml in 10 mM HEPES (Sigma-Aldrich, USA), pH 7.5, to a final concentration of 1 mg/ml. First, a three-fold molar excess of NHS-phosphine (Thermo Scientific Pierce, USA) was added to the TuMV solution, followed by overnight incubation in the dark at 4 °C. Then, the excess of NHS-phosphine was eliminated with the supernatant obtained by centrifugation (50 min, 60,000 x g, 4 °C). After washing the pellet with 10 mM HEPES, it was resuspended to a final concentration of 1 mg/ml. EGF (Agrenvec, Spain) was resuspended according to manufacturer instructions. A three-fold molar NHS-azide linker (Sigma-Aldrich, USA) was added to the EGF solution, followed by overnight incubation at 4°C. The unattached linker was eliminated by filtration with an Amicon (Merck, USA). A three-fold molar excess of EGF-azide was added to TuMV-phosphine and incubated

overnight in the dark at 4 °C. Excess EGF-azide was removed by ultracentrifugation (50 min, 60,000 x g, 4 °C). TuMV-EGF was stored at 4 °C until further use.

Dot-blot. To determine if the attachment of EGF to TuMV was successful, 10 µl of samples were added to two dry polyvinylidene difluoride (PVDF) membranes and let to completely absorb. Next, both membranes were placed on blocking solution (2% (w/v) non-fat milk in PBS-Tween20 0.05% (v/v)) overnight at 4 °C. After the blocking was completed, membranes were washed by three 5-minute baths in PBS-Tween20 5% (v/v). One membrane was immersed in 1:1000 anti-TuMV antibody (Agdia, USA) solution for 1 hour at room temperature and washed. The second membrane was immersed in a 1:500 Anti-EGF antibody (Agrenvec, Spain) solution for 1 hour at room temperature and washed. Subsequently, both membranes were immersed in a 1:50000 alkaline phosphatase anti-rabbit AP (Agdia, USA) solution for 1 hour at room temperature and washed. For visualization, both membranes were incubated in an NBT-BCIP solution at room temperature until samples were revealed.

Preparation of TuMV-EGFR-GelMA constructs. A GelMa 5% (w/v) solution with Dubelco's phosphate-buffered saline at pH 7.4 was incubated in a water bath at 37 °C until complete dissolution. Then, the photoinitiator was incorporated in a concentration of 0.067% (w/v). TuMV or TuMV-Alexa₅₅₅ was added to a final concentration of 20 µg/ml, and the resulting solution was sterile-filtered using 0.22 µm polyethersulfone (PES) filters. Drops of 30 µL of the TuMV-GelMA ink was placed on the bottom of each well of a 96-well ultra-low attachment (ULA) plate.

Evaluation of biocompatibility of TuMV-EGFR-GelMA constructs. 8000 Human fibroblasts BJ cells (ATCC® CRL-2522™) were seeded on 96-well ultra-low adherence (ULA) plate. Cell-laden constructs were monitored over a period of 7 days. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin was used for cell culture. Cells were incubated at 37 °C and 5% CO₂.

Preparation of TuMV-GelMA Bio inks. A GelMa 5% w/v solution with Dubelco's phosphate-buffered saline at pH 7.4 was incubated in a water bath at 37 °C until complete dissolution. Then, the photoinitiator was incorporated in a concentration of 0.067% w/v. TuMV or TuMV-Alexa₅₅₅ was added to a final concentration of 20 µg/ml, and the resulting solution was sterile-filtered using 0.22 µm polyethersulfone (PES) filters. TuMV-GelMA ink was placed in a 3 ml sterile cartridge and stored overnight at 4 °C.

Also, cell-laden TuMV-GelMA inks for cultures using bioprinting were produce using mouse myoblast C2C12 cells (ATCC® CRL-1772™) at 1×10^6 cells/ml. Constructs were printed using the BioX printer from Cellink® (Gothenburg, Sweden). Cell-laden printed constructs were monitored over a period of 7 days. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin was used for cell culture. Cells were incubated at 37 °C and 5% CO₂.

Live/Dead[®] staining. The medium was removed and replaced by 20 mM HEPES pH 7.1. Afterward, Live/Dead[®] (Life Technologies[™], Ireland) reagent was added to hydrogels for 20 minutes. Fluorescent images were obtained using Zeiss Axio Observer Apotome 2 (Carl Zeiss, AG, Jena, Germany) fluorescence inverted microscope.

Statistical Analysis. Statistical analysis of data was performed using Prism 9 (Graphpad).

3.3 Results and discussion

Within tissue engineering, hydrogels are widely used since they can be tunable in order to mimic the extracellular matrix (ECM). In order to achieve the latter, hydrogels must often be supplemented with either chemical or physical cues in order to reproduce tissue microenvironments (Caliari and Burdick, 2016). The addition of TuMV to GelMA represents a versatile technological platform since TuMV constitutes a modifiable scaffold.

TuMV morphology was assessed by transmission electron microscopy (TEM) and atomic force microscopy (AFM). As represented in Figure 3.2A and 3.2B, the filamentous shape of this virus resembles components of native ECM. This was expected since the resemblance has been previously studied in other plant-based VNPs scaffolds as anchoring sites for cells (Zhao, Lin and Wang, 2015). For instance, rod-like plant viruses, like Tobacco mosaic virus (TMV) have previously been shown as effective scaffolds that accelerate cell proliferation and differentiation when coated on a 2D substrate (Sitasuwan et al., 2014). This arrangement could be useful as a nanoscaffold for cells.

Figure 3. 2 | (A)AFM micrography of TuMV. (B)Schematic representation of cells over a TuMV nanomesh.
(C)TEM micrography of TuMV.

As expected, TuMV structures found in TEM (Figure 3.2C) were similar and corresponding to previous studies (Sánchez et al., 2013; González-Gamboa et al., 2017; Cuesta et al., 2019). To our knowledge, this is the first time TuMV has been visualized using AFM pictures (shown in Figure 3.2A). The fact that it can be tested through this technology opens the door for mechanical testing of the nanobiomaterial. Figure 3.2B shows a schematic representation of mammalian cells over the AFM-micrographs of TuMV to represent the scales at which these components interact.

For the evaluation of TuMV as a viable additive, we tested cell proliferation on TuMV-GelMA constructs, as depicted in Figure 3.3. Microscopy images were analyzed on days 3 and 5 (Figure 3.4). It can be observed that even with low concentrations of the virus, a higher level of cell elongation can be perceived, in comparison with the ones with GelMA.

Figure 3. 3 | GelMA hydrogels and TuMV-GelMA as cell scaffolds. (A) Graphic representation of GelMA bed for cell culture. (B) Graphic representation of cells growing over GelMA. (C) Graphic representation of GelMA enriched with TuMV (TuMV-GelMA) for cell culture. (D) Graphic representation of cells growing over TuMV-GelMA.

Figure 3. 4 | Cell adhesion and proliferation. 8000 BJ cells were seeded over GelMA, TuMV-GelMA (0.01 and 0.10 ng/ml, respectively) constructs. Observed cell adhesion and elongation on days 3 and 5.

Cell elongation and proliferation were analyzed through fluorescence stainings with Live and Dead assays at day 3 (Figure 3.5A) and PrestoBlue™ metabolic activity assay (Figure 3.5B). The fluorescent micrographs and the quantitative metabolic activity data show that the incorporation of TuMV and the change in morphology have no negative effect on the number of viable cells. This is consistent with our hypothesis that TuMV has an effect on cell-binding while not being cytotoxic.

Figure 3.5 | (A) Live and Dead micrographs on day 3. Live cells are stained green. Scale bar 200 μ m. (B)

PrestoBlue assay measuring cell viability at days 0, 1, and 3.

Cell elongation was analyzed through the calculation of the form factor using the formula $4\pi \cdot (\text{cell area} / \text{cell perimeter}^2)$. Cells that have a more rounded morphology tend to 1, while those very elongated tend to 0.01 (Figure 3.6A). Those over GelMA enriched with TuMV were significantly more elongated in comparison to GelMA without TuMV (Figure 3.6B). The latter suggests that TuMV may have an effect

on how cells anchor themselves to the scaffold. This can also be addressed by measuring the area covered by cells in the constructs. As seen in Figure 3.6 C, the area covered by cells was lower on TuMV-GelMA than with GelMA. These results are consistent with the elongation results (Figure 3.6B); cells that are more elongated cover less surface area. The addition of TuMV nanoparticles to GelMA hydrogels has a remarkable effect on the extent and rate of proliferation and the morphology of human fibroblast cells.

Figure 3. 6 | (A)Schematic representation of form factor. Form factor is defined as $4\pi \cdot (\text{cell area}/\text{cell perimeter}^2)$. Cells are considered rounder as the value of form factor approaches to 1. (B) Form factor image analysis of Live and Dead micrographs on day 3. Data represent the means and standard errors of three replicates ($p < 0.05$, t-test) (C) Image analysis of % area of cell coverage in micrographs with GelMA and TuMV-GelMA. Data represent the means and standard errors of three replicates ($p < 0.05$, t-test).

Functionalization of TuMV for cell proliferation

Hydrogels can be functionalized with signaling molecules or biological factors to promote the growth and maturation of specific tissues. For instance, growth factors (GFs) are naturally occurring proteins that stimulate cellular proliferation and differentiation. Application of GFs in a clinical setting is limited due to their short effective half-life, low stability, and rapid inactivation by enzymes under physiological conditions (Wang *et al.*, 2017).

We use TuMV VNPs, functionalized with GF, for proliferation enhancement within GelMA-based hydrogels. The structure of the only protein of the TuMV capsid was recently elucidated (Cuesta *et al.*, 2019). The most readily available amino acids are lysines, which are projected on the VNP surface, as shown schematically in Figure 3.7.

Figure 3. 7 | Scheme of the TuMV bioconjugation reaction to tether EGF via a Staudinger reaction between phosphine and azide.

Lysine abundance makes this residue the best candidate to obtain a high degree of derivatization, even more than 2000 theoretical units of the conjugated

compound. In this case, the group chosen for conjugation with lysine groups was an NHS ester since these esters generate very stable amide bonds (Hermanson, 2013). The Staudinger reaction (Figure 3.7) was chosen to get a spontaneous conjugation under physiological conditions by phosphine-azide linkage.

A first approach to validate the successful TuMV-EGF conjugation consisted of a Dot Blot assay, which was performed on a nitrocellulose membrane. Two different types of antibodies were used, namely an anti-EGF or an anti-TuMV antibody. As expected, droplets (with free or conjugated EGF) reacted with anti-EGF antibodies, while those with free or conjugated TuMV exhibited a colorimetric reaction to anti-TuMV antibodies (Figure 3.8A). To validate that the conjugation did not alter the morphology and self-assembly of the virion, electron transmission microscopy was used (Figure 3.8B). No apparent differences in size (width/length) and morphology between the conjugated virions and the WT were found. This suggests that the conjugation did not change the virion flexuous characteristic. Previous reports of conjugations to TuMV are consistent with these observations (González-Gamboa *et al.*, 2017).

Figure 3. 8 | (A)Immunoassay (Dot-Blot) to verify the TuMV-EGF conjugation; with anti-EGF antibody and anti-TuMV antibody. (B) Morphology of TuMV nanofibers visualized by TEM (left); and morphology of the EGF-conjugated nanoscaffold visualized by TEM (right). Scale bars 200 nm.

Cells exposed to TuMV, EGF and TuMV-EGF were assayed to determine their adhesion and proliferation according to each treatment. We observed enhanced rates of cell adhesion and proliferation during the first 72h in fibroblast cultures conducted in both 2D and TuMV-EGF-GelMA hydrogels, as compared to those observed in cultures on TuMV-GelMA, and GelMA added with EGF-enriched culture medium (Figure 3.9A). A cell viability assay was performed on the constructs. The LIVE / DEAD® reagent was used to discriminate between living and dead cells. This assay was carried out after 72 h culture. Cells per mm² and area covered by cells in these three treatments were analyzed from the Live and Dead micrographies (Figure 3.9B and Figure 3.9C respectively). Higher coverage was observed in GelMA enriched with TuMV-EGF (Figure 3.9B). Results suggest that GelMA-TuMV-EGF treatments accelerated cell proliferation. Consistently, fibroblasts grown on TuMV-EGF-GelMA substrates showed greater spreading and proliferation compared to aggregates with free EGF. The physical interplay between the mesh and cells has not been characterized in this work. However, it is interesting, that after the observed initial

boost, cells interacting with adsorbed TuMV-EGF continued to proliferate rapidly. The multimeric presentation of EGF to the surface of the virion enhanced cell proliferation, making it more available for cell attachment and spreading. These results suggest that TuMV not only serves as a nanocarrier for EGF but also functions as a physical-mechanical cue for cells. The viral nanomesh might be interacting with the yet-to-be adhered cells in the medium and probably attaching to them, making the EGF effect last longer.

Figure 3. 9 | (A) Live and Dead assay: fluorescence micrographs of fibroblasts attached on EGF-GelMA, GelMA with EGF, GelMA-TuMV, or GelMA-TuMV-EGF substrates after 72-h seeding. Live cells are stained green, dead cells are stained red. Scale bars of 200 μ m. (B) Cell count of live cells vs. dead cells. Data represent the means and standard errors of three replicates ($p < 0.05$, One-way ANOVA). (C) Image analysis of % area of cell coverage in micrographs with TuMV-GelMA, EGF-GelMA and TuMV-EGF-GelMA. Data represent the means and standard errors of three replicates ($p < 0.05$, * $p < 0.05$, ** $p < 0.005$, One-way ANOVA followed by Tukey's multiple comparison test).

GelMA-TuMV hydrogels used in biofabrication

Biofabrication methods require materials with specific properties. When these materials are enhanced with NPs, their properties are modified, and it is crucial to ensure that the material is still suitable for the intended biofabrication method to be used. We explored different manufacturing strategies and cell lines to prove the suitability of our VNPs-based ink in the engineered tissues.

The first biofabrication strategy explored was the manufacturing of GelMA disks adsorbed with TuMV nanoparticles and then cultured with murine fibroblast cells on their surface (Figure 3.10A). As seen in Figure 3.10B, EGF-conjugated TuMV added to GelMA provided a robust scaffold in which cells could proliferate and potentially create thick tissues (1.6 mm) in a matter of a few days (7 days). This is of vital importance in tissue engineering; it is not easy to obtain this cell density in small areas, using hydrogels as scaffolding. Confluent disc manufacturing protocols would allow us in the future to obtain even thicker constructs by simply stacking discs.

Figure 3. 10 | (A) Hydrogel discs in ULA plates (i) Schematic representation of disc fabrication, (ii) Live and Dead assay of cell disk at day 7 post-seeding. Scale bar 500 µm.

The other tested technique is the use of a 3D extrusion bioprinter. Bioprinting is an emerging biofabrication technology capable of manufacturing engineered tissues with complex three-dimensional architectures. Gelatin methacryloyl (GelMA) hydrogels are viewed as ideal bioink matrices for bioprinting due to their versatility in biomedical applications and their cost-effectiveness (Yue et al, 2015). GelMA inks can be tuned for a diverse range of applications, such as bone, cartilage, skin, etc.

TuMV nanoparticles were conjugated with Alexa Fluor 555 to facilitate their visualization of the virus distribution within GelMA hydrogels (Figure 3.11 A). TuMV-Alexa₅₅₅ was mixed with GelMA and loaded to a printer cartridge. As seen in Figure 3.11 B, the TuMV-Alexa₅₅₅ construct was found throughout the cross-section of a printed fiber. This was particularly interesting since the dispersion of TuMV-Alexa₅₅₅ particles may facilitate its access to cells within the construct that were dispersed throughout the construct (Figure 3.11 C). In order to observe cell distribution after 21 days, C2C12 cells within these constructs were stained with an F-Actin stain (red) and DAPI (blue) in order to visualize filamentous structures of the cytoskeleton and nucleus accordingly. As seen in Figure 3.11 D, C2C12 cells were able to grow within the constructs. The latter is promising since the addition of TuMV-Alexa₅₅₅ not only did not hinder the process of fiber bioprinting, but also promoted cell proliferation.

Figure 3. 11 | (A) Schematic representation of the functionalization of TuMV with Alexa Fluor 555, (B) schematic representation of the use of functionalized TuMV-based inks for 3D printing. (C) C2C12 cells in a TuMV-GelMA scaffold. Scale bar 500 μm . (D) C2C12 cells in a TuMV- scaffold after 21 days. Cells were stained with F-actin (red) and DAPI (blue). Scale bar 200 μm .

We aimed to generate functional nanostructured micro-scaffolds through the extrusion of a bioink containing TuMV NPs and C2C12 cells embedded in GelMA. The printability of our bioinks was assessed by performing 3D printing experiments using a BioX (CellInk, Sweden) bioprinter equipped with a cooling printhead and evaluating three different extrusion pressures. As shown in Figure 3.12A, we used four ink-formulations and single lines were printed to assess the fidelity of the experimental structures as compared to the CAD design. The TuMV NPs had the same level of fidelity as the pristine samples.

Thicknesses of the fibers were more consistent at a 30 kPa extrusion pressure. The addition of TuMV and/or cells did not significantly change the fidelity at the different tested pressures. Overall, constructs produced with the TuMV enriched bioink showed lower deviations on varying pressures (Figure 3.12B). Cells showed a more aligned and spread morphology in TuMV-GelMA than in pristine GelMA bioinks. This suggests that TuMV provides a suitable nanostructured micro-scaffold for muscle attachment and alignment.

Figure 3. 12 | (A) Images of the printed constructs in ultra-low adherence (ULA) plates. (B) Thickness analysis of the printed constructs. Data represent the means and standard errors of one set of fifteen replicates, and (C) variation of volume fraction through the thickness of a fiber as calculated by image analysis.

3.4 Conclusions

Here, we introduce the use of TuMV as a versatile additive for GelMA-based bioinks. TuMV serves as nano-topographical micro-scaffold for cells and provides a protein framework for further biological-specific functionalization. The presence of TuMV in the hydrogel did not hinder cell proliferation.

The presence of TuMV-EGF on the surface of hydrogels, even at relatively low concentrations (0.10 $\mu\text{g/ml}$), promoted the accelerated proliferation of fibroblasts. This resulted in the development of tissue constructs with high degrees of confluence.

In this work we also introduce the possibility of manufacturing relatively thick tissue constructs (in the range of 100 microns to 1.5 mm), which would solve one of the main current challenges of tissue engineering.

The TuMV-GelMA bioinks showed a satisfactory printability when fabricating a different array of constructs using a commercial bioprinter. Our work illustrates a strategy to develop tailored hydrogels for controlled growth factor release applications using plant viral nanomeshes as nanostructured scaffolds. These TuMV-enriched hydrogels have the potential to be applied in medical scenarios such as regenerative treatments for burn injuries, post-surgery incisions, and chronic wounds.

Future work will include the customization of TuMV-GelMA inks for different applications through the exploitation of the bioconjugation of the TuMV capsid with bioactive molecules or peptides. Evaluations of the immunogenicity of TuMV-GelMA composites in animal models, as well as determining the mechanism(s) and rate(s) of *in vivo* TuMV-GelMA degradation/absorption, are relevant goals for determining future biomedical applications.

GENERAL DISCUSSION

Evaluation of TuMV biotechnological applications

From a biomaterial perspective, *Turnip mosaic virus* is a promising standpoint for biotechnological applications. TuMV is assembled by identical CP protein and forms a highly homogenous nanostructure with a filamentous symmetry (Cuenca *et al.*, 2016; Le *et al.*, 2017). In specific, VNPs are a promising subclass of nanocarriers for drug delivery since they have demonstrated effectiveness in this application (Shukla *et al.*, 2013; Wen and Steinmetz, 2016; Finbloom *et al.*, 2018; Pitek *et al.*, 2018). One of the most interesting advantages of this type of nanoparticle is the vast array of methodologies in which it can be modified.

In the present work we were able to functionalize TuMV with different of molecules (Alexa₅₅₅, Cy_{5.5}, EGCG and EGF) in order to cater for different objectives. Throughout this thesis TuMV was tested as a luminous probe in mouse models, drug carrier and biomaterial for tissue engineering. The latter attest for the versatility of TuMV as a technological platform.

The tunable properties of TuMV in conjunction with the biocompatibility makes this biomaterial a promising candidate for applications in the biomedical field. Plant-derived virus nanoparticles have also demonstrated better cytotoxicity profiles and are degradable on biological conditions (Shukla *et al.*, 2013; Czapar and Steinmetz, 2017). The latter was also found in present results, TuMV did not show acute cytotoxicity in all tested cells lines. Furthermore, there was a clear interaction of the cells with TuMV-Alexa₅₅₅ that was observable outside and inside the cell.

When TuMV was injected to an *in vivo* model. TuMV-AlexaCy_{5.5} decayed over time, which is an indication of tissue clearance, which is consistent with tumor homing (Shukla *et al.*, 2015). As expected, TuMV-Alexa₅₅₅ was accumulated in the liver and in tumor tissue. This shows that TuMV symmetry is also a benefit to consider since it has been reported that flexuous rods allow deeper penetration into tumor tissue (Shukla *et al.*, 2013). These characteristics show potential for TuMV as an imaging tool that can be useful for diagnostics.

Functionalization of TuMV derived VNPs with plant polyphenols

The incorporation of EGCG into nanoparticles is a promising strategy to help the bioavailability and, as a result, opens the path for the exploitation of its many beneficial activities. In spite of the numerous health-promoting activities its poor bioavailability limits its applications. This is another instance in which the symmetry of TuMV can be used as an advantage since the multimeric display of EGCG improved its antimicrobial activities while prolonging the time in which these activities can be seen since these VNPs retained antimicrobial activities 6 months after their production.

Antimicrobial, antitumor and antiviral activities of EGCG-TuMV were tested but there are additional health benefits associated to this polyphenolic molecule that can be explored. For example, antioxidant activities of EGCG has been linked in the

aid of wound healing and the prevention of cardiovascular and neurodegenerative diseases (Huang *et al.*, 2019; Zagury, Kazir and Livney, 2019). In order to determine if antioxidant activities of EGCG are retained when presented in a nanoparticle, additional tests are needed. As seen in the present results, antimicrobial and antitumor activities were preserved and improved while antiviral activities were impaired. The latter highlights that the potential benefits depend on the desired applications.

TuMV as a technological platform may offer additional possibilities since it is possible to do multiple functionalization and fabrication of biomaterials. The addition of another molecule besides EGCG to the surface of TuMV offers the possibilities of either improving stability, or health-promoting activities. Cancer treatment using doxorubicin and EGCG diminished some of the negative secondary effects of this anticancer drug (Yao *et al.*, 2017; Wang *et al.*, 2019). On the other hand, hydrogels have been widely used for on demand drug delivery (Hu *et al.*, 2017; Dai *et al.*, 2018). It has been reported that formulated hydrogels with EGCG showed antimicrobial properties and biocompatibility with potential in treatment of inflammatory diseases (Dai *et al.*, 2018; Hu *et al.*, 2018). EGCG-TuMV has the potential in a number of possible applications that can be further applied in the fabrication of new biomaterials while enhancing health promoting activities such as antimicrobial and antitumor.

Bionanotechnology for tissue engineering: development of gelatin methacryloyl (GelMA) enriched with TuMV-derived nanoparticles.

Hydrogels resemble some of the properties of the extracellular matrix in mammals and have become a key biomaterial in the tissue engineering arena. These water-swollen polymeric scaffolds are frequently added with chemical and physical cues to more closely recapitulate real tissue microenvironments. Here, we propose a plant-based viral nanoparticle (VNP), the Turnip mosaic virus (TuMV), as a promising additive for the engineering of mammalian tissues. These VNPs exhibit several advantages: they are incapable of infecting mammalian cells, and their protein-based capsid allows for chemical conjugation to provide further tissue-specific chemical stimulation. We introduce the use of TuMV as a versatile additive for GelMA-based bioinks. TuMV serves as nano-topographical micro-scaffold for cells and provides a protein framework for further biological-specific functionalization. The presence of TuMV in the hydrogel did not hinder cell proliferation.

The presence of TuMV-EGF on the surface of hydrogels, even at relatively low concentrations (0.10 $\mu\text{g/ml}$), promoted the accelerated proliferation of fibroblasts. This resulted in the development of tissue constructs with high degrees of confluence.

In this work we also introduce the possibility of manufacturing relatively thick tissue constructs (in the range of 100 microns to 1.5 mm). The TuMV-GelMA bioinks

showed a satisfactory printability when fabricating a different array of constructs using a commercial bioprinter. Our work illustrates a strategy to develop tailored hydrogels for controlled growth factor release applications using plant viral nanomeshes as nanostructured scaffolds. These TuMV-enriched hydrogels have the potential to be applied in medical scenarios such as regenerative treatments for burn injuries, post-surgery incisions, and chronic wounds.

Future work will include the customization of TuMV-GelMA inks for different applications through the exploitation of the bioconjugation of the TuMV capsid with bioactive molecules or peptides. Evaluations of the immunogenicity of TuMV-GelMA composites in animal models, as well as determining the mechanism(s) and rate(s) of *in vivo* TuMV-GelMA degradation/absorption, are relevant goals for determining future biomedical applications.

CONCLUSIONS

The results obtained in this thesis allow to conclude:

1. Heat treatment of TuMV at 50 °C for 15 minutes allowed infection inactivation while largely maintaining virion structure
2. There was no apparent toxicity when TuMV is introduced to HeLa, MCF7, mesenchymal stem cells, Caco-2, Bt-474, SKBR-3, A-459, SiHa cell cultures in concentrations 0.01 – 0.10 mg/ml
3. TuMV interacted with HeLa, MCF7, MCF10A, and Caco-2 cell membrane and cells were able to take up TuMV
4. In vivo models showed that TuMV was accumulated in liver and tumor tissue after intravenous injection
5. It was possible to attach EGCG via Mannich reaction to the surface of TuMV
6. TuMV-EGCG maintained and enhanced antitumor and antimicrobial activities
7. TuMV-EGCG enhanced the inhibition of biofilm formation of *P. aeruginosa*
8. TuMV-EGCG retained antimicrobial activity *in vivo* in a plant model
9. TuMV-EGCG hindered EGCG interaction with ZIKV which limited antiviral activities
10. TuMV-GelMa formulations were suitable for cell culture and attachment promoting elongation
11. TuMV-GelMa formulations were suitable for 3-D bioprinting
12. It was possible to attach EGF to the surface of TuMV, retaining its cell proliferation activity

REFERENCES

- Albrecht, D. S. et al. (2008) 'Epigallocatechin-3-gallate (EGCG) inhibits PC-3 prostate cancer cell proliferation via MEK-independent ERK1/2 activation', *Chemico-Biological Interactions*, 171(1), pp. 89–95. doi: 10.1016/j.cbi.2007.09.001.
- Alemzadeh, E. et al. (2018) 'Plant virus nanoparticles: Novel and robust nanocarriers for drug delivery and imaging', *Colloids and Surfaces B: Biointerfaces*. Elsevier B.V., 167, pp. 20–27. doi: 10.1016/j.colsurfb.2018.03.026.
- Almatrood, S. A. et al. (2020) 'Potential therapeutic targets of epigallocatechin gallate (EGCG), the most abundant catechin in green tea, and its role in the therapy of various types of cancer', *Molecules*, 25(14). doi: 10.3390/molecules25143146.
- Anselmo, A. C. and Mitragotri, S. (2015) 'A Review of Clinical Translation of Inorganic Nanoparticles', *The AAPS Journal*, 17(5), pp. 1041–1054. doi: 10.1208/s12248-015-9780-2.
- Aslam, B. et al. (2018) 'Antibiotic resistance: a rundown of a global crisis', *Infection and Drug Resistance*, Volume 11, pp. 1645–1658. doi: 10.2147/IDR.S173867.
- Babich, H. et al. (2005) 'Differential in vitro cytotoxicity of (–)-epicatechin gallate (ECG) to cancer and normal cells from the human oral cavity', *Toxicology in Vitro*, 19(2), pp. 231–242. doi: 10.1016/j.tiv.2004.09.001.
- Babu, P. V. A. and Liu, D. (2009a) 'Flavonoids and Cardiovascular Health', in *Complementary and Alternative Therapies and the Aging Population*. Elsevier, pp. 371–392. doi: 10.1016/B978-0-12-374228-5.00018-4.
- Babu, P. V. A. and Liu, D. (2009b) 'Flavonoids and Cardiovascular Health', in *Complementary and Alternative Therapies and the Aging Population*. Elsevier, pp. 371–392. doi: 10.1016/B978-0-12-374228-5.00018-4.
- Babu, P. V. A. and Liu, D. (2009c) 'Flavonoids and Cardiovascular Health', *Complementary and Alternative Therapies and the Aging Population*, pp. 371–392. doi: 10.1016/B978-0-12-374228-5.00018-4.
- Bäcker, M. et al. (2017) 'Tobacco mosaic virus as enzyme nanocarrier for electrochemical biosensors', *Sensors and Actuators, B: Chemical*. Elsevier B.V., 238, pp. 716–722. doi: 10.1016/j.snb.2016.07.096.
- Bahadar, H. et al. (2016) 'Toxicity of nanoparticles and an overview of current experimental models', *Iranian Biomedical Journal*, 20(1), pp. 1–11. doi:

10.7508/ibj.2016.01.001.

Baião, D. et al. (2017) 'Polyphenols from Root, Tubercles and Grains Cropped in Brazil: Chemical and Nutritional Characterization and Their Effects on Human Health and Diseases', *Nutrients*, 9(9), p. 1044. doi: 10.3390/nu9091044.

Bárcena, J. and Blanco, E. (2013) 'Design of novel vaccines based on virus-like particles or chimeric virions', *Subcellular Biochemistry*. doi: 10.1007/978-94-007-6552-8_21.

Benjamin, C. et al. (2020) 'Virus like particles: fundamental concepts, biological interactions, and clinical applications', in *Nanoparticles for Biomedical Applications*. Elsevier, pp. 153–174. doi: 10.1016/B978-0-12-816662-8.00011-4.

Beutler, J. A. (2019) 'Natural Products as a Foundation for Drug Discovery', *Current Protocols in Pharmacology*, 86(1), p. e67. doi: 10.1002/cpph.67.

Blanco, A. R. et al. (2005) 'Epigallocatechin gallate inhibits biofilm formation by ocular staphylococcal isolates', *Antimicrobial Agents and Chemotherapy*, 49(10), pp. 4339–4343. doi: 10.1128/AAC.49.10.4339-4343.2005.

Brigger, I., Dubernet, C. and Couvreur, P. (2002) 'Nanoparticles in cancer therapy and diagnosis', *Advanced Drug Delivery Reviews*. doi: 10.1016/S0169-409X(02)00044-3.

Browne, S. et al. (2018) 'TGF- β 1/CD105 signaling controls vascular network formation within growth factor sequestering hyaluronic acid hydrogels', *PLoS ONE*, 13(3), pp. 1–17. doi: 10.1371/journal.pone.0194679.

Buzea, C., Pacheco, I. I. and Robbie, K. (2007) 'Nanomaterials and nanoparticles: Sources and toxicity', *Biointerphases*, 2(4), pp. MR17–MR71. doi: 10.1116/1.2815690.

Byv, W. (2012) 'Closteroviridae', in *Virus Taxonomy*. Elsevier, pp. 987–1001. doi: 10.1016/B978-0-12-384684-6.00085-9.

Caliari, S. R. and Burdick, J. A. (2016) 'A practical guide to hydrogels for cell culture', *Nature Methods*. doi: 10.1038/nmeth.3839.

Calland, N. et al. (2012) '(-)-Epigallocatechin-3-gallate is a new inhibitor of hepatitis C virus entry', *Hepatology*. doi: 10.1002/hep.24803.

Cárdenas-Vargas, A. et al. (2016) 'Evaluation of the immunogenicity of a potyvirus-like particle as an adjuvant of a synthetic peptide', *Viral Immunology*, 29(10), pp. 557–564. doi: 10.1089/vim.2016.0087.

Carneiro, B. M. et al. (2016) 'The green tea molecule EGCG inhibits Zika virus entry', *Virology*. Elsevier, 496, pp. 215–218. doi: 10.1016/j.virol.2016.06.012.

Chandra, G. K. et al. (2012) 'Interaction of (-)-epigallocatechin gallate with lysozyme-conjugated silver nanoparticles', *Applied Spectroscopy*, 66(7), pp. 744–749. doi: 10.1366/11.06533.

Chattopadhyay, S. et al. (2017) 'Nanoparticle Vaccines Adopting Virus-like Features for Enhanced Immune Potentiation', *Nanotheranostics*, 1(3), pp. 244–260. doi: 10.7150/ntno.19796.

Chavva, S. R. et al. (2019) 'Epigallocatechin gallate-gold nanoparticles exhibit superior antitumor activity compared to conventional gold nanoparticles: Potential synergistic interactions', *Nanomaterials*, 9(3), pp. 1–16. doi: 10.3390/nano9030396.

Chellaram, C. et al. (2014) 'Significance of Nanotechnology in Food Industry', *APCBEE Procedia*. Elsevier B.V., 8(Caas 2013), pp. 109–113. doi: 10.1016/j.apcb.2014.03.010.

Chen, L., Remondetto, G. E. and Subirade, M. (2006) 'Food protein-based materials as nutraceutical delivery systems', *Trends in Food Science and Technology*. doi: 10.1016/j.tifs.2005.12.011.

Chen, Xiaoqiang et al. (2019) 'Effects of Tea-Polysaccharide Conjugates and Metal Ions on Precipitate Formation by Epigallocatechin Gallate and Caffeine, the Key Components of Green Tea Infusion', *Journal of Agricultural and Food Chemistry*, 67(13), pp. 3744–3751. doi: 10.1021/acs.jafc.8b06681.

Clark, K. J. et al. (1998) 'An in vitro study of theaflavins extracted from black tea to neutralize bovine rotavirus and bovine coronavirus infections', *Veterinary Microbiology*. doi: 10.1016/S0378-1135(98)00242-9.

Coates, A. et al. (2002) 'The future challenges facing the development of new antimicrobial drugs', *Nature Reviews Drug Discovery*, 1(11), pp. 895–910. doi: 10.1038/nrd940.

Cuenca, S. et al. (2016) 'Nanonets derived from turnip mosaic virus as scaffolds for increased enzymatic activity of immobilized *Candida antarctica* lipase B', *Frontiers in Plant Science*, 7(APR2016), pp. 1–9. doi: 10.3389/fpls.2016.00464.

Cuesta, R. et al. (2019) 'Structure of Turnip mosaic virus and its viral-like particles', *Scientific Reports*, 9(1), p. 15396. doi: 10.1038/s41598-019-51823-4.

Cui, Y. et al. (2012) 'AFM study of the differential inhibitory effects of the green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) against Gram-positive and Gram-negative bacteria', *Food Microbiology*, 29(1), pp. 80–87. doi: 10.1016/j.fm.2011.08.019.

Czapar, A. E. and Steinmetz, N. F. (2017) 'Plant viruses and bacteriophages for drug delivery in medicine and biotechnology', *Current Opinion in Chemical Biology*, 38(1), pp. 108–116. doi: 10.1016/j.cbpa.2017.03.013.

Dai, T. et al. (2018) 'A Nanocomposite Hydrogel with Potent and Broad-Spectrum Antibacterial Activity', *ACS Applied Materials and Interfaces*. doi: 10.1021/acsami.8b02527.

Das, S. et al. (2014) 'Antimicrobial potential of epigallocatechin-3- gallate (EGCG): a green tea polyphenol', *Journal of Biochemical and Pharmacological Research*, 2(3), pp. 167–174. Available at: <http://www.researchpub.org/journal/jbpr/jbpr.html>.

DeBerardinis, R. J. et al. (2008) 'The Biology of Cancer: Metabolic Reprogramming Fuels Cell Growth and Proliferation', *Cell Metabolism*, 7(1), pp. 11–20. doi: 10.1016/j.cmet.2007.10.002.

Dedeo, M. T., Finley, D. T. and Francis, M. B. (2011) *Viral capsids as self-assembling templates for new materials*. 1st edn, *Progress in Molecular Biology and Translational Science*. 1st edn. Elsevier Inc. doi: 10.1016/B978-0-12-415906-8.00002-9.

Dickmeis, C. et al. (2015) 'Production of hybrid chimeric PVX particles using a combination of TMV and PVX-based expression vectors', *Frontiers in Bioengineering and Biotechnology*, 3(NOV), pp. 1–12. doi: 10.3389/fbioe.2015.00189.

Douglas, T. (2006) 'Viruses: Making Friends with Old Foes', *Science*, 312(5775), pp. 873–875. doi: 10.1126/science.1123223.

Du, G.-J. et al. (2012) 'Epigallocatechin Gallate (EGCG) Is the Most Effective Cancer Chemopreventive Polyphenol in Green Tea', *Nutrients*, 4(11), pp. 1679–1691. doi: 10.3390/nu4111679.

Duffy, M. R. et al. (2009) 'Zika Virus Outbreak on Yap Island, Federated States of Micronesia', *New England Journal of Medicine*. doi: 10.1056/nejmoa0805715.

Duval, F. et al. (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. doi: 10.1155/2016/6597970.

El-Sayed, N. S. et al. (2019) 'Development of electrical conducting nanocomposite based on carboxymethyl cellulose hydrogel/silver nanoparticles@polypyrrole', *Synthetic Metals*. Elsevier, 250(March), pp. 104–114. doi: 10.1016/j.synthmet.2019.03.010.

Esquinas-Alcázar, J. (2005) 'Protecting crop genetic diversity for food security: Political, ethical and technical challenges', *Nature Reviews Genetics*. doi: 10.1038/nrg1729.

Finbloom, J. A. et al. (2018) 'Evaluation of three morphologically distinct virus-like particles as nanocarriers for convection-enhanced drug delivery to glioblastoma', *Nanomaterials*, 8(12). doi: 10.3390/nano8121007.

Flemming, H. C. et al. (2016) 'Biofilms: An emergent form of bacterial life', *Nature Reviews Microbiology*. Nature Publishing Group, 14(9), pp. 563–575. doi: 10.1038/nrmicro.2016.94.

Friedman, M. (2007) 'Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavonoids and teas', *Molecular Nutrition and Food Research*. doi: 10.1002/mnfr.200600173.

Fukai, K., Ishigami, T. and Hara, Y. (1991) 'Antibacterial Activity of Tea Polyphenols against Phytopathogenic Bacteria', *Agricultural and Biological Chemistry*. doi: 10.1271/bbb1961.55.1895.

Gadhve, K. R. et al. (2019) 'A non-persistent aphid-transmitted Potyvirus differentially alters the vector and non-vector biology through host plant quality manipulation', *Scientific Reports*, 9(1), pp. 1–12. doi: 10.1038/s41598-019-39256-5.

Gergerich, R. C. and Dolja, V. V. (2006) 'Introduction to Plant Viruses, the Invisible Foe.', *The Plant Health Instructor*. doi: 10.1094/phi-i-2006-0414-01.

Gilis, D. et al. (2001) 'Optimality of the genetic code with respect to protein stability and amino-acid frequencies.', *Genome biology*. doi: 10.1186/gb-2001-2-11-research0049.

Gogos, A., Knauer, K. and Bucheli, T. D. (2012) 'Nanomaterials in plant protection and fertilization: Current state, foreseen applications, and research priorities', *Journal of Agricultural and Food Chemistry*, 60(39), pp. 9781–9792. doi: 10.1021/jf302154y.

González-Gamboa, I. et al. (2017) 'Plant-made potyvirus-like particles used for log-increasing antibody sensing capacity', *Journal of Biotechnology*. Elsevier, 254(June), pp. 17–24. doi: 10.1016/j.jbiotec.2017.06.014.

Gopal, J. et al. (2016) 'Bactericidal activity of green tea extracts: the importance of catechin containing nano particles', *Scientific Reports*. Nature Publishing Group, 6(1), p. 19710. doi: 10.1038/srep19710.

Grasso, S. et al. (2013) 'A plant derived multifunctional tool for nanobiotechnology based on Tomato bushy stunt virus', *Transgenic Research*, 22(3), pp. 519–535. doi: 10.1007/s11248-012-9663-6.

Gupta, R. and Xie, H. (2018) 'Nanoparticles in Daily Life: Applications, Toxicity and Regulations', *Journal of Environmental Pathology, Toxicology and Oncology*, 37(3), pp. 209–230. doi: 10.1615/JEnvironPatholToxicolOncol.2018026009.

Gutiérrez-Bugallo, G. et al. (2019) 'Vector-borne transmission and evolution of Zika virus', *Nature Ecology and Evolution*. doi: 10.1038/s41559-019-0836-z.

Habtemariam, S. (2002) 'Hamamelitannin from *Hamamelis virginiana* inhibits the tumour necrosis factor- α (TNF)-induced endothelial cell death in vitro', *Toxicon*, 40(1), pp. 83–88. doi: 10.1016/S0041-0101(01)00195-7.

Hagerman, A. E. (2012) 'Fifty Years of Polyphenol-Protein Complexes', in V'eronique Cheynier, Pascale Sarni-Manchado, and St'ephane Quideau (eds) *Recent Advances in Polyphenol Research, Volume 3*. 1st edn. Oxford, UK: Wiley-Blackwell, pp. 71–97. doi: 10.1002/9781118299753.ch3.

Hartmann, R. et al. (2012) 'Chapter 7 - Investigating Nanoparticle Internalization Patterns by Quantitative Correlation Analysis of Microscopy Imaging Data', in Nanoscience, J. M. de la F. and V. G. B. T.-F. of (ed.) *NanobiotechnologyInorganic Nanoparticles vs Organic Nanoparticles*. Elsevier, pp. 181–196. doi: <https://doi.org/10.1016/B978-0-12-415769-9.00007-8>.

Hassan, M. et al. (2019) '3D printing of biopolymer nanocomposites for tissue engineering: Nanomaterials, processing and structure-function relation', *European Polymer Journal*. Elsevier Ltd, 121, p. 109340. doi: 10.1016/j.eurpolymj.2019.109340.

Hastak, K. et al. (2003) 'Role of p53 and NF- κ B in epigallocatechin-3-gallate-induced apoptosis of LNCaP cells', *Oncogene*, 22(31), pp. 4851–4859. doi: 10.1038/sj.onc.1206708.

Hengge, R. (2019) 'Targeting bacterial biofilms by the green tea polyphenol EGCG', *Molecules*, 24(13), pp. 15–17. doi: 10.3390/molecules24132403.

Hermanson, G. T. (2013) *Bioconjugate Techniques: Third Edition*, *Bioconjugate Techniques: Third Edition*. doi: 10.1016/C2009-0-64240-9.

Hesketh, E. L. et al. (2018) 'The 3.3 Å structure of a plant geminivirus using cryo-EM', *Nature Communications*. Springer US, 9(1), pp. 1–10. doi: 10.1038/s41467-018-04793-6.
Ho, H. Y. et al. (2009) 'Antiviral effect of epigallocatechin gallate on enterovirus 71', *Journal of Agricultural and Food Chemistry*, 57(14), pp. 6140–6147. doi: 10.1021/jf901128u.

Holzinger, M., Goff, A. Le and Cosnier, S. (2014) 'Nanomaterials for biosensing applications: A review', *Frontiers in Chemistry*. doi: 10.3389/fchem.2014.00063.

Hu, B. et al. (2018) 'Polyphenol-Binding Amyloid Fibrils Self-Assemble into Reversible Hydrogels with Antibacterial Activity', *ACS Nano*. doi: 10.1021/acsnano.7b08969.

Hu, J. et al. (2017) 'A thermo-degradable hydrogel with light-tunable degradation and drug release', *Biomaterials*. doi: 10.1016/j.biomaterials.2016.10.015.

- Huang, C.-H. et al. (2009) 'EGCG inhibits protein synthesis, lipogenesis, and cell cycle progression through activation of AMPK in p53 positive and negative human hepatoma cells', *Molecular Nutrition & Food Research*, 53(9), pp. 1156–1165. doi: 10.1002/mnfr.200800592.
- Huang, Y. W. et al. (2019) 'Wound healing can be improved by (2)-epigallocatechin gallate through targeting Notch in streptozotocin-induced diabetic mice', *FASEB Journal*, 33(1), pp. 953–964. doi: 10.1096/fj.201800337R.
- Hui, X. et al. (2017) 'Antimicrobial mechanism of epigallocatechin gallate and gallic acid: They target 1-deoxy-D-xylulose 5-phosphate reductoisomerase, the key enzyme of the MEP terpenoid biosynthetic pathway', *Archives of Biochemistry and Biophysics*. Elsevier Inc, 622, pp. 1–8. doi: 10.1016/j.abb.2017.04.007.
- Hulin, M. T. et al. (2018) 'Comparative genomics of *Pseudomonas syringae* reveals convergent gene gain and loss associated with specialization onto cherry (*Prunus avium*)', *New Phytologist*. doi: 10.1111/nph.15182.
- Hulkoti, N. I. and Taranath, T. C. (2014) 'Biosynthesis of nanoparticles using microbes-A review', *Colloids and Surfaces B: Biointerfaces*. Elsevier B.V., 121, pp. 474–483. doi: 10.1016/j.colsurfb.2014.05.027.
- Hull, R. et al. (2016) *Comparative Plant Virology*, Elsevier. doi: 10.1017/CBO9781107415324.004.
- Ibort, P. (2012) 'Turnip mosaic virus-based nanoplatforms for applications in nanobiotechnology', pp. 1–14.
- Ibrahim, A., Odon, V. and Kormelink, R. (2019) 'Plant Viruses in Plant Molecular Pharming: Toward the Use of Enveloped Viruses', *Frontiers in Plant Science*, 10(June), pp. 1–18. doi: 10.3389/fpls.2019.00803.
- Ikigai, H. et al. (1993) 'Bactericidal catechins damage the lipid bilayer', *BBA - Biomembranes*, 1147(1), pp. 132–136. doi: 10.1016/0005-2736(93)90323-R.
- Isaacs, C. E. et al. (2011) 'Digallate dimers of (-)-epigallocatechin gallate inactivate herpes simplex virus', *Antimicrobial Agents and Chemotherapy*. doi: 10.1128/AAC.05531-11.
- ISHII, T. et al. (2011) 'Human Serum Albumin as an Antioxidant in the Oxidation of (-)-Epigallocatechin Gallate: Participation of Reversible Covalent Binding for Interaction and Stabilization', *Bioscience, Biotechnology, and Biochemistry*, 75(1), pp. 100–106. doi: 10.1271/bbb.100600.

Jeevanandam, J. et al. (2018) 'Review on nanoparticles and nanostructured materials: history, sources, toxicity and regulations', *Beilstein Journal of Nanotechnology*, 9(1), pp. 1050–1074. doi: 10.3762/bjnano.9.98.

Jeon, J. et al. (2014) 'The antimicrobial activity of (-)-epigallocatechin-3-gallate and green tea extracts against *Pseudomonas aeruginosa* and *Escherichia coli* isolated from skin wounds', *Annals of Dermatology*, 26(5), pp. 564–569. doi: 10.5021/ad.2014.26.5.564.

Jha, A. K. et al. (2015) 'Molecular weight and concentration of heparin in hyaluronic acid-based matrices modulates growth factor retention kinetics and stem cell fate', *Journal of Controlled Release*. Elsevier B.V., 209, pp. 308–316. doi: 10.1016/j.jconrel.2015.04.034.

Jiang, Y.-Y. et al. (2017) 'Preparation and enhanced mechanical properties of hybrid hydrogels comprising ultralong hydroxyapatite nanowires and sodium alginate', *Journal of Colloid and Interface Science*. Elsevier Inc., 497, pp. 266–275. doi: 10.1016/j.jcis.2017.03.032.

Jobsri, J. et al. (2015) 'Plant Virus Particles Carrying Tumour Antigen Activate TLR7 and Induce High Levels of Protective Antibody', *PLOS ONE*. Edited by S. J. Turner, 10(2), p. e0118096. doi: 10.1371/journal.pone.0118096.

Johnson, T. A. et al. (2016) 'Clusters of antibiotic resistance genes enriched together stay together in swine agriculture', *mBio*. doi: 10.1128/mBio.02214-15.

Keller, A. A. et al. (2017) 'Comparative environmental fate and toxicity of copper nanomaterials', *NanoImpact*. Elsevier B.V., 7, pp. 28–40. doi: 10.1016/j.impact.2017.05.003.

Koudelka, K. J. et al. (2015) 'Virus-Based Nanoparticles as Versatile Nanomachines', *Annual Review of Virology*, 2(1), pp. 379–401. doi: 10.1146/annurev-virology-100114-055141.

Kruk, T. et al. (2015) 'Synthesis and antimicrobial activity of monodisperse copper nanoparticles', *Colloids and Surfaces B: Biointerfaces*, 128, pp. 17–22. doi: 10.1016/j.colsurfb.2015.02.009.

Kumar, N. B. et al. (2016) 'Randomized, placebo-controlled trial evaluating the safety of one-year administration of green tea catechins', *Oncotarget*, 7(43), pp. 70794–70802. doi: 10.18632/oncotarget.12222.

Kümmerer, K. et al. (2011) 'Biodegradability of organic nanoparticles in the aqueous environment', *Chemosphere*. doi: 10.1016/j.chemosphere.2010.11.069.

- Lamichhane, J. R. et al. (2015) 'Challenges and opportunities for integrated pest management in Europe: A telling example of minor uses', *Crop Protection*, 74, pp. 42–47. doi: 10.1016/j.cropro.2015.04.005.
- Le, D. H. T. et al. (2017) 'Chemical addressability of potato virus X for its applications in bio/nanotechnology', *Journal of Structural Biology*, 200(3), pp. 360–368. doi: 10.1016/j.jsb.2017.06.006.
- Lei, F. et al. (2014) 'Preparation and functional evaluation of chitosan-EGCG conjugates', *Journal of Applied Polymer Science*, 131(3), pp. 1–8. doi: 10.1002/app.39732.
- Leo, E. et al. (1997) 'Doxorubicin-loaded gelatin nanoparticles stabilized by glutaraldehyde: Involvement of the drug in the cross-linking process', *International Journal of Pharmaceutics*. doi: 10.1016/S0378-5173(97)00149-X.
- Li, J. et al. (2020) 'Antiviral Effect of Epigallocatechin Gallate via Impairing Porcine Circovirus Type 2 Attachment to Host Cell Receptor', *Viruses*, 12(2), p. 176. doi: 10.3390/v12020176.
- Li, Q. et al. (2007) 'Morphology and stability changes of recombinant TMV particles caused by a cysteine residue in the foreign peptide fused to the coat protein', *Journal of Virological Methods*, 140(1–2), pp. 212–217. doi: 10.1016/j.jviromet.2006.10.011.
- Li, Z. et al. (2014a) 'Fabrication of coated bovine serum albumin (BSA)-epigallocatechin gallate (EGCG) nanoparticles and their transport across monolayers of human intestinal epithelial Caco-2 cells', *Food and Function*, 5(6), pp. 1278–1285. doi: 10.1039/c3fo60500k.
- Li, Z. et al. (2014b) 'Fabrication of coated bovine serum albumin (BSA)-epigallocatechin gallate (EGCG) nanoparticles and their transport across monolayers of human intestinal epithelial Caco-2 cells', *Food & Function*, 5(6), p. 1278. doi: 10.1039/c3fo60500k.
- Lico, C., Chen, Q. and Santi, L. (2008) 'Viral vectors for production of recombinant proteins in plants', *Journal of Cellular Physiology*, 216(2), pp. 366–377. doi: 10.1002/jcp.21423.
- Liu, Y. and Chan-Park, M. B. (2010) 'A biomimetic hydrogel based on methacrylated dextran-graft-lysine and gelatin for 3D smooth muscle cell culture', *Biomaterials*, 31(6), pp. 1158–1170. doi: 10.1016/j.biomaterials.2009.10.040.
- Liu, Z. et al. (2008) 'Drug delivery with carbon nanotubes for in vivo cancer treatment', *Cancer Research*. doi: 10.1158/0008-5472.CAN-08-1468.

Lovato, A. et al. (2019) 'Inhibition of Virulence-Related Traits in *Pseudomonas syringae* pv. *actinidiae* by Gunpowder Green Tea Extracts', *Frontiers in Microbiology*, 10(October), pp. 1–17. doi: 10.3389/fmicb.2019.02362.

Luo, K.-W. et al. (2017) 'EGCG inhibited bladder cancer SW780 cell proliferation and migration both in vitro and in vivo via down-regulation of NF- κ B and MMP-9', *The Journal of Nutritional Biochemistry*. Elsevier Inc., 41, pp. 56–64. doi: 10.1016/j.jnutbio.2016.12.004.

Lyu, S. Y., Rhim, J. Y. and Park, W. B. (2005) 'Antiherpetic activities of flavonoids against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) in vitro', *Archives of Pharmacal Research*. doi: 10.1007/BF02978215.

MA, Y.-C. et al. (2014) 'Epigallocatechin gallate inhibits the growth of human lung cancer by directly targeting the EGFR signaling pathway', *Oncology Reports*, 31(3), pp. 1343–1349. doi: 10.3892/or.2013.2933.

Makkar, H. (2000) *Quantification of Tannins in Tree Foliage*. 26. Vienna.

Makkar, H. P. S., Dawra, R. K. and Singh, B. (1988) 'Determination of Both Tannin and Protein in a Tannin-Protein Complex', *Journal of Agricultural and Food Chemistry*, 36(3), pp. 523–525. doi: 10.1021/jf00081a600.

Malarkey, D. E., Hoenerhoff, M. J. and Maronpot, R. R. (2018) 'Carcinogenesis', in *Fundamentals of Toxicologic Pathology*. Elsevier, pp. 83–104. doi: 10.1016/B978-0-12-809841-7.00006-X.

Malekhosseini, P. et al. (2019) 'Development of casein-based nanoencapsulation systems for delivery of epigallocatechin gallate and folic acid', *Food Science and Nutrition*, 7(2), pp. 519–527. doi: 10.1002/fsn3.827.

Marsian, J. and Lomonossoff, G. P. (2016) 'Molecular pharming — VLPs made in plants', *Current Opinion in Biotechnology*. Elsevier Ltd, 37, pp. 201–206. doi: 10.1016/j.copbio.2015.12.007.

Martin-Acebes, M. A. et al. (2014) 'The Composition of West Nile Virus Lipid Envelope Unveils a Role of Sphingolipid Metabolism in Flavivirus Biogenesis', *Journal of Virology*, 88(20), pp. 12041–12054. doi: 10.1128/jvi.02061-14.

Mateu, M. G. (2011) 'Virus engineering: functionalization and stabilization', *Protein Engineering Design and Selection*, 24(1–2), pp. 53–63. doi: 10.1093/protein/gzq069.

Md Nesran, Z. N. et al. (2019) 'Induction of Endoplasmic Reticulum Stress Pathway by Green Tea Epigallocatechin-3-Gallate (EGCG) in Colorectal Cancer Cells: Activation of PERK/p-eIF2 α /ATF4 and IRE1 α ', *BioMed Research International*, 2019. doi: 10.1155/2019/3480569.

- Mereles, D. and Hunstein, W. (2011) 'Epigallocatechin-3-gallate (EGCG) for clinical trials: More Pitfalls than Promises?', *International Journal of Molecular Sciences*, 12(9), pp. 5592–5603. doi: 10.3390/ijms12095592.
- Middleton, E., Kandaswami, C. and Theoharides, T. C. (2000) 'The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer', *Pharmacological Reviews*.
- Moeinzadeh, S. and Jabbari, E. (2017) *Springer Handbook of Petroleum Technology*. Edited by C. S. Hsu and P. R. Robinson. Cham: Springer International Publishing (Springer Handbooks). doi: 10.1007/978-3-319-49347-3.
- Moreno, A. and Fereres, A. (2012) 'Virus Diseases in Lettuce in the Mediterranean Basin', in *Advances in Virus Research*. 1st edn. Elsevier Inc., pp. 247–288. doi: 10.1016/B978-0-12-394314-9.00007-5.
- Mottier, A. et al. (2017) 'Environmental impact of engineered carbon nanoparticles: from releases to effects on the aquatic biota', *Current Opinion in Biotechnology*, 46, pp. 1–6. doi: 10.1016/j.copbio.2016.11.024.
- Musso, D. and Gubler, D. J. (2016) 'Zika Virus', *Clinical Microbiology Reviews*, 29(3), pp. 487–524. doi: 10.1128/CMR.00072-15.
- Nadim, M. et al. (2014) 'Improvement of polyphenol properties upon glucosylation in a UV-induced skin cell ageing model', *International Journal of Cosmetic Science*, 36(6), pp. 579–587. doi: 10.1111/ics.12159.
- Naito, M. et al. (2018) *Nanoparticle Technology Handbook, Nanoparticle Technology Handbook*. doi: 10.1016/s1748-0132(07)70119-6.
- Niedzwiecki, A. et al. (2016) 'Anticancer Efficacy of Polyphenols and Their Combinations', *Nutrients*, 8(9), p. 552. doi: 10.3390/nu8090552.
- Nomura, K. et al. (2006) 'A bacterial virulence protein suppresses host innate immunity to cause plant disease', *Science*. doi: 10.1126/science.1129523.
- O'May, C. et al. (2012) 'Tannin derived materials can block swarming motility and enhance biofilm formation in *Pseudomonas aeruginosa*', *Biofouling*, 28(10), pp. 1063–1076. doi: 10.1080/08927014.2012.725130.
- Pandey, K. B. and Rizvi, S. I. (2009) 'Plant Polyphenols as Dietary Antioxidants in Human Health and Disease', *Oxidative Medicine and Cellular Longevity*, 2(5), pp. 270–278. doi: 10.4161/oxim.2.5.9498.

Papuc, C. et al. (2017) 'Plant Polyphenols as Antioxidant and Antibacterial Agents for Shelf-Life Extension of Meat and Meat Products: Classification, Structures, Sources, and Action Mechanisms', *Comprehensive Reviews in Food Science and Food Safety*, 16(6), pp. 1243–1268. doi: 10.1111/1541-4337.12298.

Patel, K. D., Singh, R. K. and Kim, H. W. (2019) 'Carbon-based nanomaterials as an emerging platform for theranostics', *Materials Horizons*. doi: 10.1039/c8mh00966j.

Pelgrift, R. Y. and Friedman, A. J. (2013) 'Nanotechnology as a therapeutic tool to combat microbial resistance', *Advanced Drug Delivery Reviews*. Elsevier B.V., 65(13–14), pp. 1803–1815. doi: 10.1016/j.addr.2013.07.011.

Petersen, L. R. et al. (2016) 'Zika Virus', *New England Journal of Medicine*. Edited by L. R. Baden, 374(16), pp. 1552–1563. doi: 10.1056/NEJMr1602113.

Pitek, A. S. et al. (2018) 'Cancer Theranostic Applications of Albumin-Coated Tobacco Mosaic Virus Nanoparticles', *ACS Applied Materials & Interfaces*, 10(46), pp. 39468–39477. doi: 10.1021/acsami.8b12499.

Pokorski, J. K. and Steinmetz, N. F. (2011) 'The art of engineering viral nanoparticles', *Molecular Pharmaceutics*, 8(1), pp. 29–43. doi: 10.1021/mp100225y.

Potenza, M. A. et al. (2020) 'The intrinsic virtues of EGCG, an extremely good cell guardian, on prevention and treatment of diabetes complications', *Molecules*, 25(13). doi: 10.3390/molecules25133061.

Price, W. C. (1964) 'Inactivation and Denaturation of Plant Viruses', in *Journal of Chemical Information and Modeling*, pp. 171–217. doi: 10.1016/S0065-3527(08)60699-5.

Rady, I. et al. (2018) 'Cancer preventive and therapeutic effects of EGCG, the major polyphenol in green tea', *Egyptian Journal of Basic and Applied Sciences*. Mansoura University, 5(1), pp. 1–23. doi: 10.1016/j.ejbas.2017.12.001.

Ramachandran, B. et al. (2016) 'Repeated dose studies with pure Epigallocatechin-3-gallate demonstrated dose and route dependant hepatotoxicity with associated dyslipidemia', *Toxicology Reports*. Elsevier Ireland Ltd, 3, pp. 336–345. doi: 10.1016/j.toxrep.2016.03.001.

Ranzato, E. et al. (2014) 'Epigallocatechin-3-gallate elicits Ca²⁺ spike in MCF-7 breast cancer cells: Essential role of Cav3.2 channels', *Cell Calcium*. Elsevier Ltd, 56(4), pp. 285–295. doi: 10.1016/j.ceca.2014.09.002.

Rathore, K. et al. (2012) 'Green tea catechin intervention of reactive oxygen species-mediated ERK pathway activation and chronically induced breast cell carcinogenesis', *Carcinogenesis*, 33(1), pp. 174–183. doi: 10.1093/carcin/bgr244.

Relat, J. et al. (2012) 'Different fatty acid metabolism effects of (-)-Epigallocatechin-3-Gallate and C75 in Adenocarcinoma lung cancer', *BMC Cancer*, 12. doi: 10.1186/1471-2407-12-280.

Revers, F. and García, J. A. (2015a) 'Molecular biology of potyviruses', *Advances in Virus Research*, 92, pp. 101–199. doi: 10.1016/bs.aivir.2014.11.006.

Revers, F. and García, J. A. (2015b) 'Molecular Biology of Potyviruses', in *Advances in Virus Research*. 1st edn. Elsevier Inc., pp. 101–199. doi: 10.1016/bs.aivir.2014.11.006.

Riechmann, J. L., Lain, S. and Garcia, J. A. (1992) 'Highlights and prospects of potyvirus molecular biology', *Journal of General Virology*. doi: 10.1099/0022-1317-73-1-1.

Rohovie, M. J., Nagasawa, M. and Swartz, J. R. (2017) 'Virus-like particles: Next-generation nanoparticles for targeted therapeutic delivery', *Bioengineering & Translational Medicine*, 2(1), pp. 43–57. doi: 10.1002/btm2.10049.

Rooney, W. M. et al. (2020) 'Engineering bacteriocin-mediated resistance against the plant pathogen *Pseudomonas syringae*', *Plant Biotechnology Journal*, 18(5), pp. 1296–1306. doi: 10.1111/pbi.13294.

Sakamoto, Y. et al. (2013) 'Effects of Epigallocatechin-3-gallate (EGCG) on A549 Lung Cancer Tumor Growth and Angiogenesis', *Bioscience, Biotechnology, and Biochemistry*, 77(9), pp. 1799–1803. doi: 10.1271/bbb.120882.

Sánchez-Navarro, M. and Rojo, J. (2012) *Nanobiotechnology - Inorganic Nanoparticles vs Organic Nanoparticles*, *Frontiers of Nanoscience*. doi: 10.1016/B978-0-12-415769-9.00005-4.

Sánchez-Tena, S. et al. (2012) 'Hamamelitannin from Witch Hazel (*Hamamelis virginiana*) Displays Specific Cytotoxic Activity against Colon Cancer Cells', *Journal of Natural Products*, 75(1), pp. 26–33. doi: 10.1021/np200426k.

Sánchez, F. et al. (2013) 'Plant viral elongated nanoparticles modified for log-increases of foreign peptide immunogenicity and specific antibody detection', *Journal of Biotechnology*. Elsevier B.V., 168(4), pp. 409–415. doi: 10.1016/j.jbiotec.2013.09.002.

Sánchez, F. and Ponz, F. (2018) 'Viruses and Plant Development', in R. Gaur, Khurana, S., and Dorokhov, Y. (eds) *Plant Viruses. Diversity, Interaction and Management*. 1 st. Boca Ratón: CRC Press, pp. 267–278.

Santamaría-Hernando, S. et al. (2018) '*Pseudomonas syringae* pv. tomato exploits light signals to optimize virulence and colonization of leaves', *Environmental Microbiology*, 20(12), pp. 4261–4280. doi: 10.1111/1462-2920.14331.

Scida, K. et al. (2011) 'Recent applications of carbon-based nanomaterials in analytical chemistry: Critical review', *Analytica Chimica Acta*, 691(1–2), pp. 6–17. doi: 10.1016/j.aca.2011.02.025.

Scofield, J. and Wu, H. (2019) 'Microbial biofilms', in *Encyclopedia of Microbiology*. doi: 10.1016/B978-0-12-801238-3.99205-5.

Shankar, S., Suthakar, G. and Srivastava, R. K. (2007) 'Epigallocatechin-3-gallate inhibits cell cycle and induces apoptosis in pancreatic cancer', *Frontiers in Bioscience*, 12(12), p. 5039. doi: 10.2741/2446.

Shtay, R. et al. (2019) 'Encapsulation of (–)-epigallocatechin-3-gallate (EGCG) in solid lipid nanoparticles for food applications', *Journal of Food Engineering*. Elsevier B.V., 244, pp. 91–100. doi: 10.1016/j.jfoodeng.2018.09.008.

Shukla, S. et al. (2013) 'Increased Tumor Homing and Tissue Penetration of the Filamentous Plant Viral Nanoparticle Potato virus X', *Molecular Pharmaceutics*, 10(1), pp. 33–42. doi: 10.1021/mp300240m.

Shukla, S. et al. (2014) 'Molecular farming of fluorescent virus-based nanoparticles for optical imaging in plants, human cells and mouse models', *Biomaterials Science*, 2(5), pp. 784–797. doi: 10.1039/c3bm60277j.

Shukla, S. et al. (2015) 'To Target or Not to Target: Active vs. Passive Tumor Homing of Filamentous Nanoparticles Based on Potato virus X', *Cellular and Molecular Bioengineering*, 8(3), pp. 433–444. doi: 10.1007/s12195-015-0388-5.

Silva, A. M. et al. (2019) 'Comparison of antiproliferative effect of epigallocatechin gallate when loaded into cationic solid lipid nanoparticles against different cell lines', *Pharmaceutical Development and Technology*. Taylor & Francis, 24(10), pp. 1243–1249. doi: 10.1080/10837450.2019.1658774.

Simpson, D. and Amos, S. (2017) *Other Plant Metabolites, Pharmacognosy: Fundamentals, Applications and Strategy*. Elsevier Inc. doi: 10.1016/B978-0-12-802104-0.00012-3.

Singh, B. N., Shankar, S. and Srivastava, R. K. (2011) 'Green tea catechin, epigallocatechin-3-gallate (EGCG): Mechanisms, perspectives and clinical applications', *Biochemical Pharmacology*, 82(12), pp. 1807–1821. doi: 10.1016/j.bcp.2011.07.093.

Singh, T. G. et al. (2016) *Nanobiomaterials: Applications in biomedicine and biotechnology, Fabrication and Self-Assembly of Nanobiomaterials: Applications of Nanobiomaterials*. Elsevier Inc. doi: 10.1016/B978-0-323-41533-0.00013-1.

Sitasuwan, P. et al. (2014) 'RGD-conjugated rod-like viral nanoparticles on 2D scaffold improve bone differentiation of mesenchymal stem cells', *Frontiers in Chemistry*. doi: 10.3389/fchem.2014.00031.

Smith, A. et al. (2010) 'Nanolipidic particles improve the bioavailability and α -secretase inducing ability of epigallocatechin-3-gallate (EGCG) for the treatment of Alzheimer's disease', *International Journal of Pharmaceutics*. Elsevier B.V., 389(1–2), pp. 207–212. doi: 10.1016/j.ijpharm.2010.01.012.

Smith, M. T., Hawes, A. K. and Bundy, B. C. (2013) 'Reengineering viruses and virus-like particles through chemical functionalization strategies', *Current Opinion in Biotechnology*. Elsevier Ltd, 24(4), pp. 620–626. doi: 10.1016/j.copbio.2013.01.011.

Song, Q. et al. (2014) 'Enhanced uptake and transport of (+)-catechin and (-)-epigallocatechin gallate in niosomal formulation by human intestinal caco-2 cells', *International Journal of Nanomedicine*, 9(1), pp. 2157–2165. doi: 10.2147/IJN.S59331.

Spicer, C. D. and Davis, B. G. (2014) 'Selective chemical protein modification', *Nature Communications*. Nature Publishing Group, 5, pp. 1–14. doi: 10.1038/ncomms5740.

Steinmetz, N. F. (2010) 'Viral nanoparticles as platforms for next-generation therapeutics and imaging devices', *Nanomedicine: Nanotechnology, Biology and Medicine*. Elsevier Inc., 6(5), pp. 634–641. doi: 10.1016/j.nano.2010.04.005.

Stenvang, M. et al. (2016) 'Epigallocatechin gallate remodels overexpressed functional amyloids in pseudomonas aeruginosa and increases biofilm susceptibility to antibiotic treatment', *Journal of Biological Chemistry*, 291(51), pp. 26540–26553. doi: 10.1074/jbc.M116.739953.

Su, J. et al. (2020) 'Utilization of β -lactoglobulin-(-)-Epigallocatechin-3-gallate(EGCG) composite colloidal nanoparticles as stabilizers for lutein pickering emulsion', *Food Hydrocolloids*. Elsevier Ltd, 98(17), p. 105293. doi: 10.1016/j.foodhyd.2019.105293.

Sun, L. M., Zhang, C. L. and Li, P. (2011) 'Characterization, antimicrobial activity, and mechanism of a high-performance (-)-Epigallocatechin-3-gallate (EGCG)-Cull/polyvinyl alcohol (PVA) nanofibrous membrane', *Journal of Agricultural and Food Chemistry*, 59(9), pp. 5087–5092. doi: 10.1021/jf200580t.

Suresh, D. et al. (2015) 'EGCG assisted green synthesis of ZnO nanopowders: Photodegradative, antimicrobial and antioxidant activities', *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*. Elsevier B.V., 136(PC), pp. 1467–1474. doi: 10.1016/j.saa.2014.10.038.

Sutradhar, P., Saha, M. and Maiti, D. (2014) 'Microwave synthesis of copper oxide nanoparticles using tea leaf and coffee powder extracts and its antibacterial activity', *Journal of Nanostructure in Chemistry*, 4(1), pp. 4–9. doi: 10.1007/s40097-014-0086-1.

Tarhini, M., Greige-Gerges, H. and Elaissari, A. (2017) 'Protein-based nanoparticles: From preparation to encapsulation of active molecules', *International Journal of Pharmaceutics*. Elsevier B.V., 522(1–2), pp. 172–197. doi: 10.1016/j.ijpharm.2017.01.067.

Tauber, A. L., Schweiker, S. S. and Levonis, S. M. (2020) 'From tea to treatment; epigallocatechin gallate and its potential involvement in minimizing the metabolic changes in cancer', *Nutrition Research*. Elsevier Inc, 74, pp. 23–36. doi: 10.1016/j.nutres.2019.12.004.

Taylor, P. W., Hamilton-Miller, J. M. T. and Stapleton, P. D. (2005) 'Antimicrobial properties of green tea catechins', *Food Science & Technology Bulletin: Functional Foods*, 2(7), pp. 71–81. doi: 10.1616/1476-2137.14184.

Thérien, A. et al. (2017) 'A versatile papaya mosaic virus (PapMV) vaccine platform based on sortase-mediated antigen coupling', *Journal of Nanobiotechnology*. BioMed Central, 15(1), p. 54. doi: 10.1186/s12951-017-0289-y.

Thomas, P. et al. (2015) 'Optimization of single plate-serial dilution spotting (SP-SDS) with sample anchoring as an assured method for bacterial and yeast cfu enumeration and single colony isolation from diverse samples', *Biotechnology Reports*. Elsevier B.V., 8, pp. 45–55. doi: 10.1016/j.btre.2015.08.003.

Tighe-Neira, R. et al. (2018) 'Metallic nanoparticles influence the structure and function of the photosynthetic apparatus in plants', *Plant Physiology and Biochemistry*. Elsevier, 130(May), pp. 408–417. doi: 10.1016/j.plaphy.2018.07.024.

Tomihama, T., Nishi, Y. and Arai, K. (2007) 'Epigallocatechin gallate, a major tea catechin, induces biofilm formation of *Pseudomonas syringae* pv. theae', *Journal of General Plant Pathology*. doi: 10.1007/s10327-007-0005-0.

Touriño, A. et al. (2008) 'High expression of foreign proteins from a biosafe viral vector derived from Turnip mosaic virus', *Spanish Journal of Agricultural Research*, 6(SPEC. ISS.), pp. 48–58. doi: 10.5424/sjar/200806s1-373.

Tripathi, S. et al. (2018) 'Nano-bioremediation: Nanotechnology and bioremediation', in *Biostimulation Remediation Technologies for Groundwater Contaminants*, pp. 202–219. doi: 10.4018/978-1-5225-4162-2.ch012.

Trosko, J. E. and Kang, K. S. (2012) 'Evolution of energy metabolism, stem cells and cancer stem cells: How the warburg and barker hypotheses might be linked', *International Journal of Stem Cells*. doi: 10.15283/ijsc.2012.5.1.39.

Tsopmo, A., Awah, F. M. and Kuete, V. (2013) *Lignans and Stilbenes from African Medicinal Plants*, *Medicinal Plant Research in Africa: Pharmacology and Chemistry*. Elsevier Inc. doi: 10.1016/B978-0-12-405927-6.00012-6.

- Valli, A. et al. (2014) 'A Novel Role of the Potyviral Helper Component Proteinase Contributes To Enhance the Yield of Viral Particles', *Journal of Virology*, 88(17), pp. 9808–9818. doi: 10.1128/JVI.01010-14.
- Vázquez-Calvo, ángela et al. (2017) 'Antiviral properties of the natural polyphenols delphinidin and epigallocatechin gallate against the flaviviruses West Nile Virus, Zika Virus, and Dengue Virus', *Frontiers in Microbiology*, 8(JUL), pp. 1–8. doi: 10.3389/fmicb.2017.01314.
- Velázquez-Lam, E., Imperial, J. and Ponz, F. (2020) 'Polyphenol-Functionalized Plant Viral-Derived Nanoparticles Exhibit Strong Antimicrobial and Antibiofilm Formation Activities', *ACS Applied Bio Materials*. doi: 10.1021/acsabm.9b01161.
- Wan, Z.-L., Guo, J. and Yang, X.-Q. (2015) 'Plant protein-based delivery systems for bioactive ingredients in foods', *Food & Function*. Royal Society of Chemistry, 6(9), pp. 2876–2889. doi: 10.1039/C5FO00050E.
- Wang, J. et al. (2018) 'A prodrug of green tea polyphenol (–)-epigallocatechin-3-gallate (Pro-EGCG) serves as a novel angiogenesis inhibitor in endometrial cancer', *Cancer Letters*. Elsevier B.V., 412, pp. 10–20. doi: 10.1016/j.canlet.2017.09.054.
- Wang, X. et al. (2014) 'Covalent complexation and functional evaluation of (–)-epigallocatechin gallate and α -lactalbumin', *Food Chemistry*. Elsevier Ltd, 150, pp. 341–347. doi: 10.1016/j.foodchem.2013.09.127.
- Wang, Y. et al. (2019) 'Ultralong Circulating Lollipop-Like Nanoparticles Assembled with Gossypol, Doxorubicin, and Polydopamine via π – π Stacking for Synergistic Tumor Therapy', *Advanced Functional Materials*. doi: 10.1002/adfm.201805582.
- Wang, Zhenming et al. (2017) 'Novel biomaterial strategies for controlled growth factor delivery for biomedical applications', *NPG Asia Materials*. doi: 10.1038/am.2017.171.
- Weber, C. et al. (2000) 'Desolvation process and surface characterisation of protein nanoparticles', *International Journal of Pharmaceutics*. doi: 10.1016/S0378-5173(99)00370-1.
- Wei, R. et al. (2018) 'Suppressing glucose metabolism with epigallocatechin-3-gallate (EGCG) reduces breast cancer cell growth in preclinical models', *Food & Function*. Royal Society of Chemistry, 9(11), pp. 5682–5696. doi: 10.1039/C8FO01397G.
- Wen, A. M. and Steinmetz, N. F. (2016) 'Design of virus-based nanomaterials for medicine, biotechnology, and energy', *Chemical Society Reviews*, 45(15), pp. 4074–4126. doi: 10.1039/C5CS00287G.

World Health Organization (2014) *Cancer Fact Sheet N297*, World Health Organization. doi: /entity/mediacentre/factsheets/fs297/en/index.html.

Wu (1992) 'Suppression of cell proliferation and gene expression by combinatorial synergy of EGCG, resveratrol and γ -tocotrienol in estrogen receptor-positive MCF-7 breast cancer cells', *International Journal of Oncology*, 33(4), pp. 547–557. doi: 10.3892/ijo_00000073.

Wylie, S. J. et al. (2017) 'ICTV virus taxonomy profile: Potyviridae', *Journal of General Virology*. doi: 10.1099/jgv.0.000740.

Xin, X. F., Kvitko, B. and He, S. Y. (2018) 'Pseudomonas syringae: What it takes to be a pathogen', *Nature Reviews Microbiology*. doi: 10.1038/nrmicro.2018.17.

Xue, J. et al. (2014) 'Fabrication of epigallocatechin-3-gallate nanocarrier based on glycosylated casein: Stability and interaction mechanism', *Journal of Agricultural and Food Chemistry*, 62(20), pp. 4677–4684. doi: 10.1021/jf405157x.

Yang, L. et al. (2010) 'Evaluation of enoyl-acyl carrier protein reductase inhibitors as *Pseudomonas aeruginosa* quorum-quenching reagents', *Molecules*, 15(2), pp. 780–792. doi: 10.3390/molecules15020780.

Yang, Y. and Zhang, T. (2019) 'Antimicrobial activities of tea polyphenol on phytopathogens: A review', *Molecules*, 24(4). doi: 10.3390/molecules24040816.

Yao, Y. F. et al. (2017) '(-)-Epigallocatechin-3-gallate alleviates doxorubicin-induced cardiotoxicity in sarcoma 180 tumor-bearing mice', *Life Sciences*. Elsevier Inc, 180, pp. 151–159. doi: 10.1016/j.lfs.2016.12.004.

Yildiz, I., Shukla, S. and Steinmetz, N. F. (2011) 'Applications of viral nanoparticles in medicine', *Current Opinion in Biotechnology*, 22(6), pp. 901–908. doi: 10.1016/j.copbio.2011.04.020.

Yuan, X. et al. (2019) 'Cellular Toxicity and Immunological Effects of Carbon-based Nanomaterials', *Particle and Fibre Toxicology*. Particle and Fibre Toxicology, 16(1). doi: 10.1186/s12989-019-0299-z.

Yuste-Calvo, C. et al. (2019) 'Structure-Based Multifunctionalization of Flexuous Elongated Viral Nanoparticles', *ACS Omega*, 4(3), pp. 5019–5028. doi: 10.1021/acsomega.8b02760.

Yuste Calvo, C. (2019) *Funcionalización con base estructural de nanopartículas virales derivadas del 'Virus del mosaico del nabo' con fines biotecnológicos*.

- Zagury, Y., Kazir, M. and Livney, Y. D. (2019) 'Improved antioxidant activity, bioaccessibility and bioavailability of EGCG by delivery in β -lactoglobulin particles', *Journal of Functional Foods*. doi: 10.1016/j.jff.2018.10.025.
- Zazo, H. et al. (2017) *Applications of Metallic Nanoparticles in Antimicrobial Therapy, Antimicrobial Nanoarchitectonics: From Synthesis to Applications*. Elsevier Inc. doi: 10.1016/B978-0-323-52733-0.00015-X.
- Zeng, L. et al. (2017) 'Stability of tea polyphenols solution with different pH at different temperatures', *International Journal of Food Properties*, 20(1), pp. 1–18. doi: 10.1080/10942912.2014.983605.
- Zhang, X. et al. (2012) 'Cyclin D1 degradation and p21 induction contribute to growth inhibition of colorectal cancer cells induced by epigallocatechin-3-gallate', *Journal of Cancer Research and Clinical Oncology*, 138(12), pp. 2051–2060. doi: 10.1007/s00432-012-1276-1.
- Zhang, Y. et al. (2009) 'Green tea (-)-epigallocatechin-3-gallate down-regulates VASP expression and inhibits breast cancer cell migration and invasion by attenuating Rac1 activity', *European Journal of Pharmacology*. Elsevier B.V., 606(1–3), pp. 172–179. doi: 10.1016/j.ejphar.2008.12.033.
- Zhao, X., Lin, Y. and Wang, Q. (2015) 'Virus-based scaffolds for tissue engineering applications', *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*, 7(4), pp. 534–547. doi: 10.1002/wnan.1327.
- Zhong, Y., Ma, C. M. and Shahidi, F. (2012) 'Antioxidant and antiviral activities of lipophilic epigallocatechin gallate (EGCG) derivatives', *Journal of Functional Foods*. Elsevier Ltd, 4(1), pp. 87–93. doi: 10.1016/j.jff.2011.08.003.
- Ziv, O., Avtalion, R. R. and Margel, S. (2008) 'Immunogenicity of bioactive magnetic nanoparticles: Natural and acquired antibodies', *Journal of Biomedical Materials Research - Part A*. doi: 10.1002/jbm.a.31518.