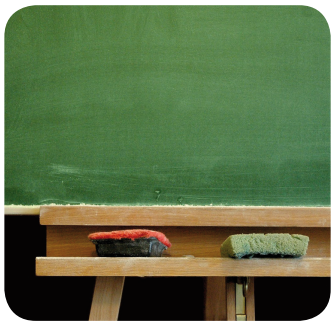

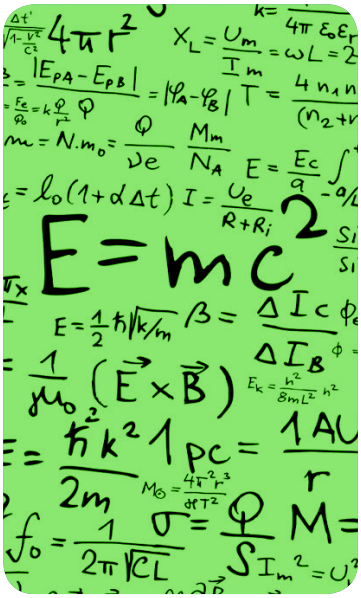

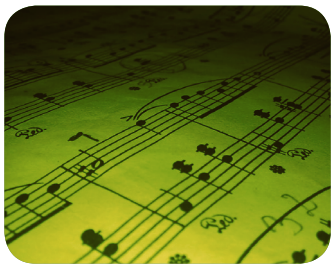

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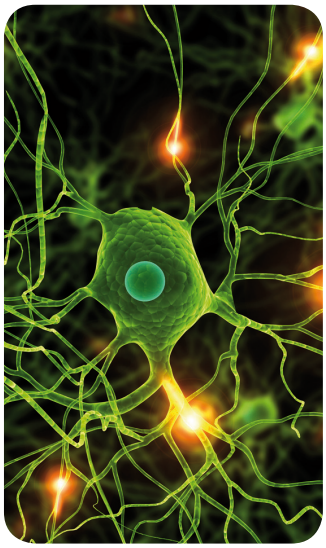

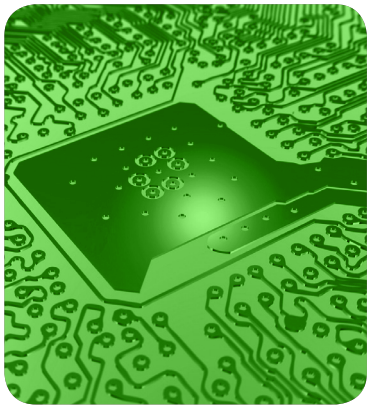

Viruses and Nanobiotechnology

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UAM EDICIONES

Viruses and Nanobiotechnology

Master Degree in Molecular Nanoscience and Nanotechnology

MSc dissertation presented by

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Department of Organic Chemistry

Madrid, April 8th, 2016

To my mother and sisters
To Adrián Guillermo and Alexander Gómez
and to Sebastián Méndez

ABBREVIATIONS

DNA = Deoxyribonucleic Acid
RNA = Ribonucleic Acid
VNP = Viral Nanoparticle
VLP = Virus-Like-Particle
CCMV = Cowpea Chlorotic Mottle Virus
CPMV = Cowpea Mosaic Virus
RCNMV = Red Clover Necrotic Mosaic Virus
TMV = Tobacco Mosaic Virus
BMV = Brome Mosaic Virus
EGFP = Green Fluorescent Protein
TYMV = Turnip Yellow Mosaic Virus
FR = Folate Receptor
HCRSV = Hibiscus Chlorotic Ringspot Virus
MRI = Magnetic Resonance Imaging
PET = Positron Emission Tomography
FITC = Fluorescein Isothiocyanate
HJV = Hemagglutinating Virus of Japan
APC = Antigen-Presenting Cell
HIV = Human Immunodeficiency Virus
PVX = Potato Virus X
TBSV = Tomato Bushy Stunt Virus
PEG = Polyethylene Glycol
FDA = Food and Drug Administration
STM = Scanning Tunneling Microscopy
AIDS = Acquired Immune Deficiency Syndrome
ELISA = Enzyme-Linked Immunosorbent Assay
PCR = Polymerase Chain Reaction
CNT = Carbon Nanotube
DHF = Dengue Hemorrhagic Fever
DSS = Dengue Shock Syndrome
NS1 = Non-Structural 1 protein
Fc = Crystallizable Fragment
MDCK = Madin-Darby Canine Kidney
HSV-1 = Herpes Virus Simple type 1
HSV-2 = Herpes Virus Simple type 2
MPP = Mucus-Penetrating Particle
HS = Heparan-Sulfate
MPV = Monkeypox Virus
HBV = Hepatitis B Virus
LHD = Layered Double Hydroxide
NF-KB = Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells
PRRS = Porcine Reproductive and Respiratory Syndrome
PRRSV = Porcine Reproductive and Respiratory Syndrome Virus
FMD = Foot-and-Mouth Disease
FMDV = Foot-and-Mouth Disease Virus
RSV = Respiratory Syncytial Virus
PST = Polysaccharide-based Polysorbitol Transporter
PEI = Polyethylenimine
MNSs= Micro-Nano Structures
gB= Glycoprotein B
RT-PCR = Retro Transcriptase Polymerase Chain Reaction

INDEX

1. Introduction

1.1 Definitions

Nano comes from the greek word *nãnos*, meaning dwarf[1]. ***Nanoscience*** is the study and the understanding of systems at the nanoscale (0.1 to 100 nm), while ***Nanotechnology*** is the control and the manipulation of them[2]. Usually, the terms are interchangeable.

According to Evans (2010), ***Nanobioscience*** is an inter-disciplinary area of research that includes Chemistry, Biology, Physics, Materials Science, Engineering and Medicine. It involves the utilization of biomaterials, devices or methodologies in which dimensions of the functional components are in the nanoscale. Nanobioscience can be divided into the use of nanotechnological devices to probe and understand biological systems, and into the exploitation of biomaterials for the design of new nanomaterials or nanodevices[1], which would refer to ***Nanobiotechnology***.

Some of the most interesting materials that are studied in this field, are viruses. Moreover, different materials can be used in the fight against viral diseases.

1.2 Viruses

Shors defines viruses in his book ‘’*Viruses: molecular study with clinic orientation*’’ (2009) as microorganisms that present the following characteristics:

- They are small, on the order of nanometers.
- They have the ability to cross bacteriological filters.
- They have dependency on a host cell.
- They have only one type of nucleic acid (DNA or RNA).
- They have receptor binding proteins.
- They have a genome that can be infectious.
- In some cases, they remain integrating its genome into a host cell.
- Viruses are inert outside their host cells. They can infect animal, plant and bacteria cells.

Viral particles have a genome surrounded by a protein structure called capsid, where adhesion proteins to the host cell reside. The union between the capsid and the genome is known as nucleocapsid, that may include viral polymerases involved in the processes of replication, transcription and reverse transcription of the virus. Furthermore, in the case of animal viruses, they may have a membrane composed of a lipid bilayer that is around the viral particle.

The entire structure of the virus, that includes the genome, capsid, and the lipid bilayer and polymerases if they exit, is known as *virion*[3].

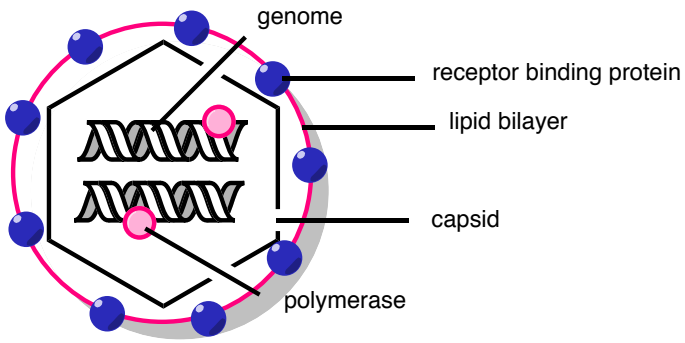

Figure 1: Structure of an animal virus.

1.3 Viruses as nanobiomaterials

The viruses can be considered nanoparticles, known as ***Viral Nanoparticles*** (VNPs). When the viruses have not genome, only a capsid, are known as ***Virus-Like-Particles*** (VLPs). The viruses that infect animals, plants and bacteria (known as bacteriophages) are used in Nanobiotechnology. However, plant viruses are the most common.

Different features make plant viruses useful as scaffolds for the synthesis of nanobiomaterials. Viruses derive from a process of self-assembly, where the capsids are very stable, and have an uniform size and shape. Characteristics of viruses can be analyzed in bulk suspensions, and capsids can be functionalized, binding different molecules and nanomaterials to them. Furthermore, plant viruses can be produced at big scale[31]. However, these characteristics could be extended to the other types of viruses.

1.4 Objective of the review

Viruses are a very promising field in Nanobiotechnology. In this review, different aspects will be treated, as the description of the most common viruses used in Nanobiotechnology, chemical modifications of them, their use in Biomedicine and Nanomedicine, and the use of nanomaterials in the fight against viral diseases and vaccine development. Therefore, the purpose of this review is to explore all these issues from a general point of view so, it can serve as a basis for the elaboration of future doctoral thesis, that could include the integration of Chemistry, Biology and Materials Science, among others disciplines.

2. Viruses used commonly in Nanobiotechnology

The C*owpea Chlorotic Mottle Virus* (CCMV), is one of the viruses used commonly in Nanobiotechnology. It is a RNA virus of the family *Bromoviridae* that infects plants. Its icosahedral capsid consists of 180 protein subunits surrounding the nucleic acid core. The capsid has a dynamic and a swelling structure. Depending on the pH and ionic strength, the 180 protein subunits can be assembled and disassembled. This can be used to remove the nucleic acid inside the capsid and replace it by different molecules. CCMV is very useful for packaging negatively charged species, because inside the capsid there is a high density of lysine and arginine residues, which are amino acids that are positively charged[6-8].

The C*owpea Mosaic Virus* (CPMV) is another plant virus of icosahedral symmetry, that belongs to the family *Comoviridae*. It is used in Nanobiotechnology due to its high stability in a wide range of temperatures, pH and buffers. On its surface are exposed lysines, which have been studied for the attack of different molecules[9,10]. The *Red Clover Necrotic Mosaic Virus* (RCNMV) is a plant virus that belongs to the family *Tombusviridae*. By varying the concentration of Ca^{2+} and Mg^{2+}, it occurs the opening of their surface pores and this can be used to pack molecules within the capsid[11].

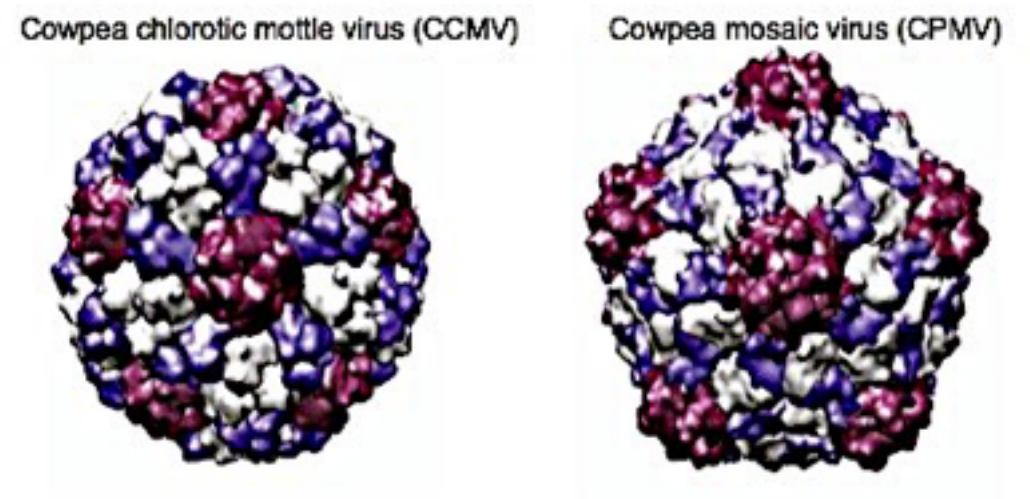

Figure 2: CCMV and CPMV, that present an icosahedral capsid. Reprinted with permission from ref 79.

The *Tobacco Mosaic Virus* (TMV), of the family *Virgaviridae*, is very stable in different chemical environments. It can also be chemically modified both externally and internally, making it another virus with a potential use in Nanobiotechnology[9,12].

MS2 phage is a bacteriophage where the nucleic acid is RNA. RNA packaging in the capsid may be performed in *Escherichia coli* strains, and is removed easily by alkaline hydrolysis leaving empty capsids[13], which could be used to pack different molecules inside. Moreover, the capsid

contains pores of 1.8 nm of diameter, which may allow access to the interior of the capsid to carry out different chemical modifications[14]. Other phages, like *Qβ* and *M13*, are widely used[6].

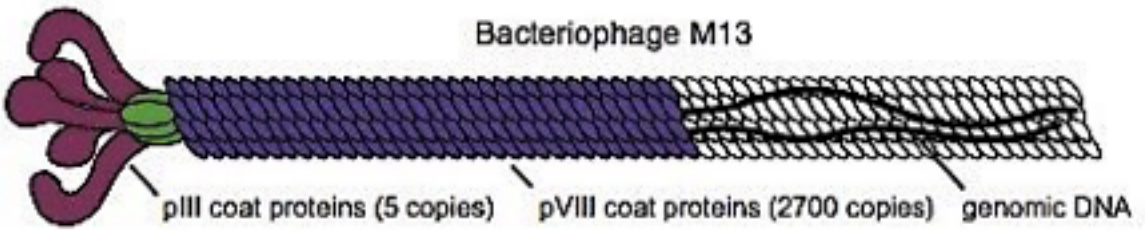

Figure 3: Bacteriophage *M13.* Reprinted with permission from ref 79.

3. Tuning the viral particles

Bioconjugation chemistry allow the functionalization of VNPs and VLPs. Small chemical modifications, such as the binding of nanoparticles or organic dyes to the capsid proteins, it is only an example. Also, this process can be controlled with an accuracy of atomic level, which is an advantage. This is critical in Viral Nanotechnology, because VNPs and VLPs constitute a transport path of many particles and molecules used in different techniques as Medical Imaging and Drug Delivery[15,16]. The exposed surface of capsids of viruses as CCMV and CPMV has a lot of amino acids that can be decorated with a large number of molecules. These amino acids are native to the virus, such as lysine, aspartic and glutamic acid. Using techniques of bioconjugation carboxi- and amino-selectives, different groups can be linked to the capsid surface[17]. For example, in CCMV for a 540 residues of lysine and 560 residues of carboxylates can be conjugated[18].

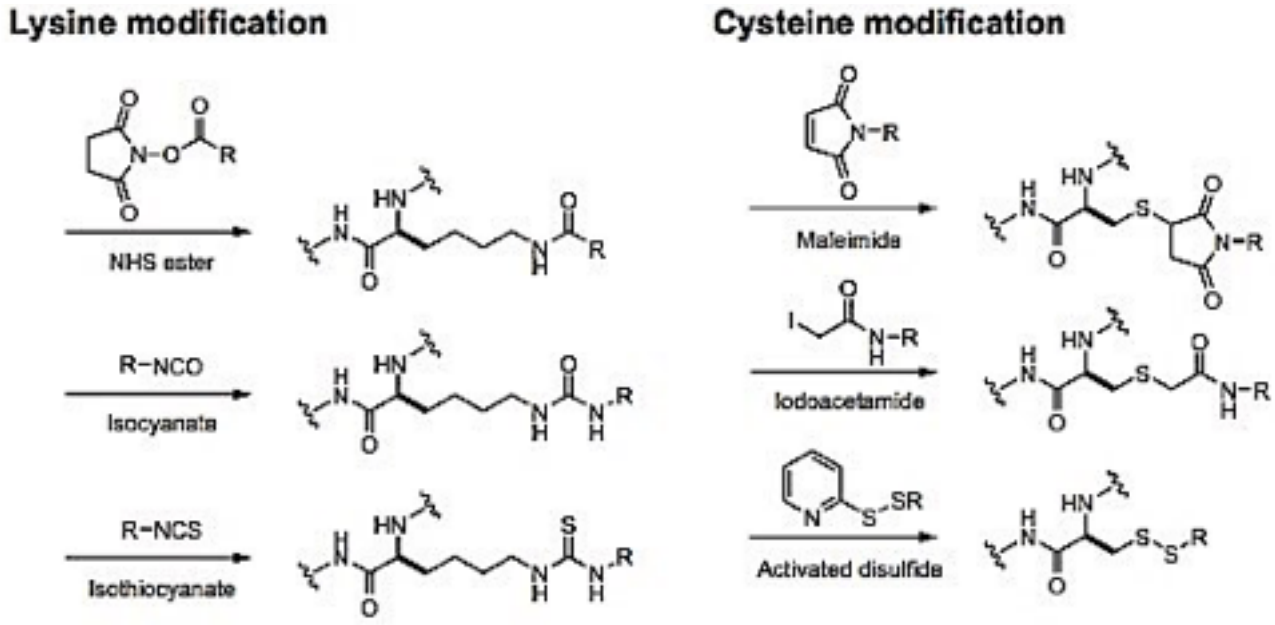

Figure 4: Chemical modifications of Lysine and Cysteine. Reprinted with permission from ref 79.

However, different modifications can be made, like introducing synthetic amino acids in a capsid. As Strable et al. (2008) report, they incorporated synthetic amino acids such as homopropargil glycine and azidohomoalanina into the capsid of VLPs replacing the amino acid methionine[19]. These substitutions had no effect on the ability of proteins to self-assemble to form the capsid.

Exploit the interactions between molecules and residues of viral capsids can be very efficient. For example, it has been possible to encapsidate drugs such as doxorubicin, in RCNMV. To pack the drug within the capsid, electrostatic interactions between the drug and the capsid surface can be achieved, and can be modulated by environmental factors such as buffer composition, pH and ionic strength[20,31].

The genetic engineering of new residues of capsids generates the appearance of multiple utilities. *Electroless deposition* is a method that has been studied widely in the mineralization of inorganic materials with biological templates[26]. The viral capsids can suffer different processes of mineralization, with the alteration of viral capsids to increase the attraction between viruses and the ions that participate in the process of mineralization. Different examples are the production of 3-D arrays of gold nanoparticles on the surface of CPMV, among many others[27-29].

Gillitzer et al. (2002) have seized bioconjugation using natural and genetically modified amino acids[18]. They have modified the outside surface of the capsid of CCMV with fluorescent molecules and small peptides. In particular, they have determined the degree of surface modification via coupling of fluorophores to lysine, glutamate and aspartate residues, and engineered cysteine residues.

Different materials can be packaged within the capsids during the self-assembly process. For example, enzymes, polymers, and nanoparticles with different properties.

In biological systems, the enzymes have their own microenvironment. If an encapsidation into VNPs or VLPs occurs, this environment can be reproduced. In addition, VNPs or VLPs containing a single enzyme may provide a model for studying the behavior of a unique enzyme [15,158].

Artificial polymers are also another option presented to pack molecules inside VNPs. VNPs encapsidate within them nucleic acids that are negatively charged, so artificial nucleic acid molecules or negatively charged polymers could be encapsidated in the same way[15].

According to Pokorski and Steinmetz (2011), synthetic nanoparticles as fluorescent quantum dots and diverse structures are useful in different techniques of Nanobiotechnology. The encapsidation of nanoparticles is a good choice to ensure biocompatibility, preventing aggregation

and allowing bioconjugation of functional ligands for a tissue specificity[15]. Chen et al. (2006) have worked with functionalized gold particles that can initiate VLP assembly of *Brome Mosaic Virus* (BMV) by mimicking the electrostatic behavior of the nucleic acid component of the native virus[21].

To pack a positively charged compound, the strategy may change. Minten et al. (2009) have achieved in one of their studies[25], the encapsidation of proteins that have a positive charge using a noncovalent anchoring.

Thus, as seen, VNPs and VLPs capsids can be chemically modified and conjugated, to be used in different nanobiotechnological applications.

4. Top-Down and Bottom-Up approaches

Evans (2010) defends that two approaches can be taken to fabricate new nanomaterials. One is the ***top-down*** approach, where a macro- or micro-scale material is machined, tooled or milled to give a material with desired properties. The other is the ***bottom-up*** approach, by which molecules or preformed precursors are brought together[1].

Bottom-up and top-down approaches can be used to build complex structures for different applications in the development of new materials.

Whitesides and Boncheva (2002) define *Molecular Self-assembly* as a process in which molecules, or parts of them, form aggregates spontaneously without human intervention. In the process, bonds are formed of a non-covalent type, from a less ordered state to a higher order one[63].

The head-to-tail assembly of TMV is a very interesting example of self-assembly in nature. The assembly of the particle can be viewed as a group of rings which are placed around a single strand of RNA that guides, forming a helical rod[64]. In solution, for the process of head-to-tail assembly of the wild-type of TMV participate a lot of conditions, like hydrophobic interactions, an acid pH and repulsions between residues. The fibers can become disassociated, but this can be solved by coating the surface of the virus with different materials, for example, through sol-gel reactions[65-67].

Modifying the self-assembly process of VNPs, to create VLPs, can be very useful. For example, the assembly of CCMV can be modulated by a semiconductor polyanionic polymer having negative charge, that mimics the negative charge of the nucleic acid. The ionic strength of the solution is used to modulate the polymer structure[77].

The technique called *Pickering Emulsion* allows the 2-D particle assembly at the interface of two immiscible liquids[68]. It is a dynamic process that allows less energy in the self-assembly process. The main factors that influence the control of this process is the particle size and surface properties. Viruses as TMV, CPMV and *Turnip Yellow Mosaic Virus* (TYMV) have been studied in these processes. For example, monolayers have been manufactured with TYMV packaged in a interface of water/perfluorodecalin, and the self-assembly of TMV has been achieved at oil/water interfaces[65,69-71].

However, while making thin layers of viruses is simple, transferring them to a solid substrate may become complicated. *Direct coating methods*, *drop and dry methods* and *dip coating methods* are the most conventional methods to produce thin films of viruses on substrates. A strong electrostatic attraction between the surface and viruses is an advantage for the formation of the layers. Other factors such as the concentration of viruses, ionic strength and pH of the solution are critical. An alternative method is the direct surface coating by *layer by layer* technique, which allows a greater control of coating viruses in terms of morphology and coverage, and characteristics of the layers can be studied using different techniques. For example, thin layers of CPMV have been built in cell cultures and they are biologically active[65,72-74].

Meanwhile, icosahedral viruses represent highly uniform 3-D blocks that allow the assembly of different structures[31]. Also, liquid crystals of viruses as templates have been designed[30].

TMV1cys is a mutant of TMV, where a cysteine codon has been incorporated, which allows the attachment of the virus to a variety of surfaces and substrates[31]. This chimeric VNP can be employed in different techniques, like *photolithographic patterning*[75]. Also, with the technique of *electron-beam lithography*, VNPs have been patterned in nanostructures[76]. These are only two examples of the Top-Down approach.

The purpose of chemical modifications made to VNPs and VLPs capsids, and the different bottom-up and top-down approaches, is the development of new and advanced techniques in Biomedicine and Nanomedicine as Medical Imaging, Drug Delivery and Photodynamic Therapy. Also, VNPs and VLPs can be used as vaccines against different diseases.

5. Viruses and Nanotechnological applications used in Biomedicine and Nanomedicine

Drug Delivery systems, Medical Imaging, Photodynamic Therapy and virus-based vaccines are a great promise of success in Biomedicine and Nanomedicine.

5.1. Drug delivery

Drug delivery systems relates to the group of approaches, techniques, formulations and systems for transporting a pharmaceutical compound throughout the body, for a particular desired therapeutic effect[22]. Futhermore, *Targeted Drug Delivery* expands the field of Drug Delivery, allowing the drug to be released into the corresponding tissues due to the presence of certain molecular receptors, thereby reducing the minimum effective dose and increasing the therapeutic efficiency [23]. It is desirable that the drug was active in a particular physiological time, implying a temporal modulation and a controlled release thereof[24]. Moreover, this helps and enhances the delivery of a drug that has a short half life[36].

The capsid of viruses that carry drugs can be modified in order to add molecules that targets in certain tissues, and the genome of the viruses can be eliminated in order to make them non-infectious. Also, capsids can be functionalized with different materials in order to make them more stable under different conditions.

Sometimes it is not necessary to modify the viral capsid to direct a VNP or a VLP to a tissue. For example, CPMV is a VNP which has a natural affinity for endothelial cells in mammals. This interaction is biospecific and it is mediated by vimentin protein in mammals, which is an intermediate filament that is expressed mostly in the cytosol of cells, but also appears on the cell surface. By this way, different types of tumors associated with these cells could be fight off. Moreover, the presence of CPMV has been detected in inflammatory lesions containing macrophages, microglia and immunoglobulins, in the central nervous system. This may provide opportunities for the specific treatment of inflammatory diseases of the central nervous system[5, 32-35].

Folic acid is another molecule that has been used for Targeted Drug Delivery to fight off cancer cells. Folic acid is an extremely important molecule in development and growth processes as cell division and DNA synthesis. The molecule links to FR, a membrane receptor, which mediates endocytosis and thus internalization. In normal cells, the expression levels of FR receptor are low, while levels in cancer cells are over expressed, i.e. cancer cells such as ovarian and uterine mesothelium. Folate can be conjugated to *Hibiscus Chlorotic Ringspot Virus* (HCRSV), binding it to surface exposed lysines, and the drug doxorubicin can be packaged into the VNP, and cancer cells can be attacked selectively[6,37-39]. The lactobionic acid has also been used for Targeted Drug Delivery, by bioconjugation to *rotavirus VP6* capsid, to combat hepatocyte cancer cells, because they have receptors for lactobionic acid[40].

Human transferrin is a glycoprotein that acts as a carrier of iron, which is essential to processes of iron homeostasis. There is a study in which this protein is applied as an element of targeting. As this protein is recognized by a receptor which is over expressed on the surface of a variety of tumor cells, it has been linked to the capsid of virus as CPMV and *Qβ,* and cancer cells could be attacked selectively if viruses transport drugs[6,41-43].

Moreover, antibodies may act as targeting molecules too. A single chain antibody which recognizes carcinoembryonic antigen, a surface protein over expressed in a variety of tumor cells, has been linked to CPMV capsids[44].

There are studies of VNPs and VLPs genetically engineered, to transport and release drugs. For example, a chimeric *Polyomavirus* expressing a Z domain in its capsid, from the protein A of *Staphylococcus aureus*, was used for linking an antibody for Targeted Drug Delivery into ErbB2-positive tumor cells, that have receptors of the tyrosine kinase type[45].

5.2. Medical Imaging

Besides Drug Delivery systems, an important field in Biomedicine and Nanomedicine is ***Medical Imaging.*** According to Kulak et al. (2015), Medical Imaging refers to the different technologies used to observe different parts of the human body, in order to diagnose, monitor or treat different medical diseases. Each type of technology gives information about a particular area of the body, which is being studied, about diseases or the effectiveness of a certain treatment. Medical Imaging systems have come a long way over the years, but the technology used is expensive, so the use is limited and scientists have to make a good selection of the techniques employed[46].

VNPs and VLPs can be modified by bioconjugation using different Imaging agents such as fluorescent dyes for optical imaging, Gadolinium complexes for Magnetic Resonance Imaging (MRI), and quantum dots and metal nanoparticles for detection methods using different spectroscopic methods[47,48].

CPMV particles carrying fluorescent labels have been used for intravital vascular Imaging, in mice embryos and chicken. CPMV is internalized by endothelial cells, resulting in high resolution images[32]. VNPs or VLPs fluorescent ligands can be combined with targeting ligands to develop powerful Imaging techniques for specific diseases. If the fluorescent labels are encapsidated within VNPs or VLPs, the surface is free to be modified with targeting ligands[5]. Examples of encapsidated Imaging agents are quantum dots, that have been packaged into *SV40 virus* and have been detected by confocal microscopy[49], or indocyanine green which has been encapsidated into VLPs of BMV[50].

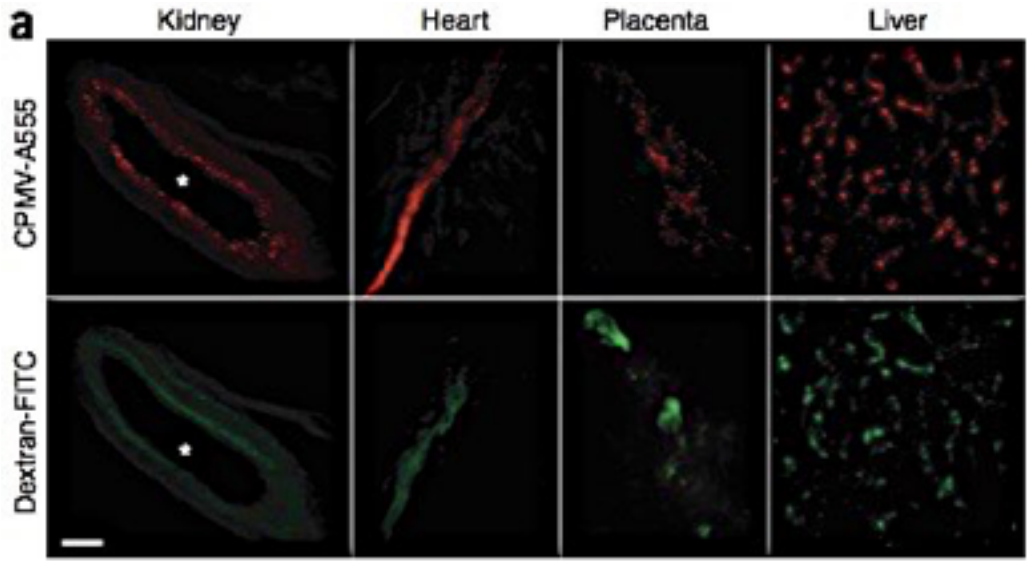

Figure 5: Fluorescence in different tissues of CPMV conjugated with A555, compared to fluorescein dextran. Reprinted with permission from ref 32.

Moreover, VNPs and VLPs can be used as a MRI contrast agent. MRI is a technique for non-invasive in vivo Imaging, based on the alignment of protons in a strong magnetic field. Contrast agents are used to increase the brightness and the sensitivity of the technique. Recently, viruses as CPMV, CCMV, MS2 and Qβ have been used as contrast agents, and Gadolinium complexes used in MRI have been linked to the capsids[5,51]. In addition, VNPs and VLPs can also carry Imaging agents for Positron Emission Tomography (PET), which is a technique that allows to view 3-D images of body processes. For example, nanoparticles of iron oxide and ^{18}F-fluoride derivatives have been encapsidated in *Hemagglutinating Virus of Japan* (HJV), which increase the PET signal in these techniques[52].

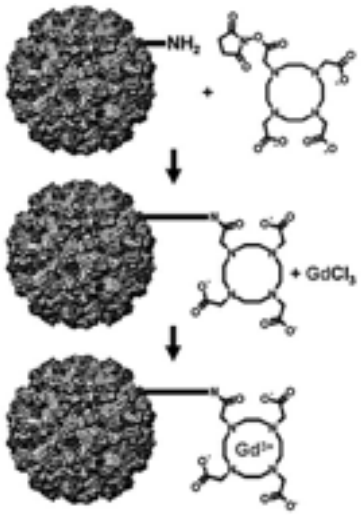

Figure 6: Scheme of the capsid of a virus, conjugated to a Gadolinium Complex. Reprinted with permission from ref 51.

5.3 Photodynamic Therapy

Photodynamic Therapy is presented as another of the major techniques used in Biomedicine and Nanomedicine. This is a technique in which a photosensitive is excited at specific wavelengths of light, to generate reactive oxygen species, which can be used to kill certain targeted cells[5]. A recent study conducted by Brasch et al. (2011) describes the encapsidation of a therapeutic agent, a zinc phthalocyanine, in CCMV. Porphyrins and phthalocyanines are agents used commonly in Photodynamic Therapy due to their extinction coefficients and efficient intersystem crossing to the triplet state. Different studies in combination with cell-targeting strategies may open up possibilities of using this system in the treatment of cancer cells[53,54,159].

Stephanopoulos et al. (2010), in this line, have created, using the bacteriophage MS2, a targeted Photodynamic Therapy vehicle for treat Jurkat leukemia T cells[78]. They modified the interior surface of the viral capsid with porphyrins, that are capable of generate singlet oxygen upon illumination, and the outside of the capsid with cell receptor-specific of DNA, that were able to target and kill Jurkat leukemia T cells.

Fullerenes and derivatives are molecules that are excellent candidates in Photodynamic Therapy too, but they present a problem, and it is its high aqueous insolubility. However, the solubility can be increased significantly through conjugation of VNPs or VLPs, like CMPV and Qβ, which act as a hydrophilic carrier and can be oriented toward target cells. In vitro studies have confirmed efficient delivery of these materials in cells[5,55].

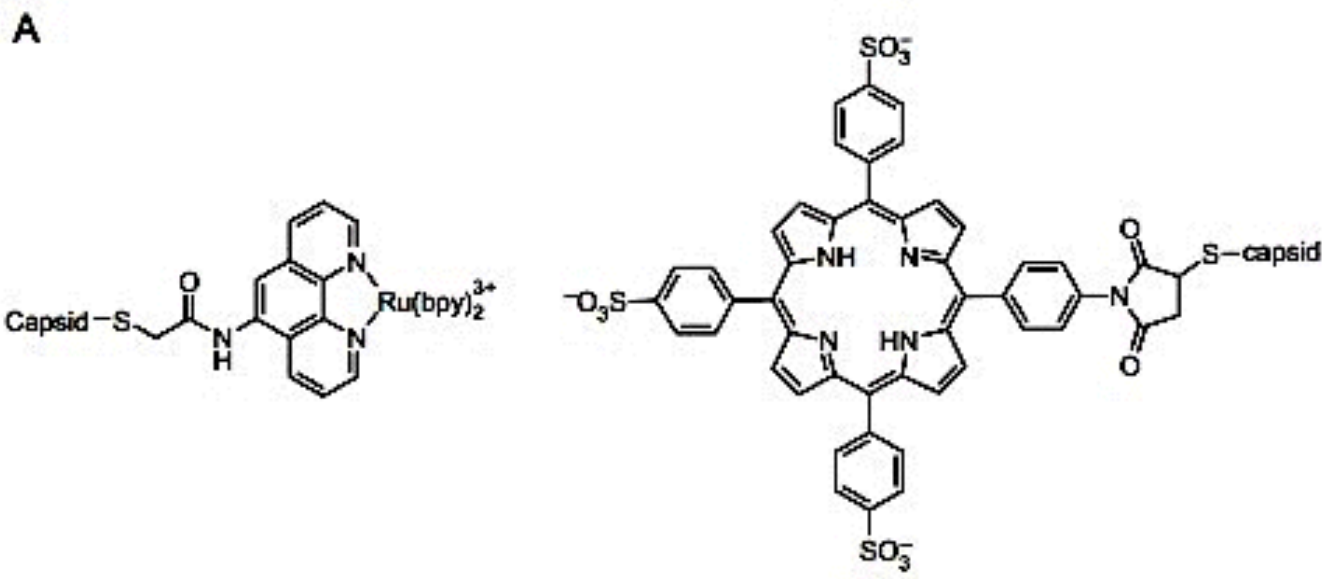

Figure 7: Generator molecules of singlet oxygen for Photodynamic Therapy. Reprinted with permission from ref 79.

5.4. Vaccine development based in VNPs and VLPs

A different trend in studies with viruses is ***vaccine development based in VNPs and VLPs***. The purpose of vaccination is to allow the development of a strong and lasting immune response against diseases. When a antigen is injected through the body, the Antigen-Presenting Cells (APCs) take the antigen, and travel to the T region of the lymphoid organs, and they present the antigen to T lymphocytes. Then, depending of generated proteins, different cell responses can be generated: the humoral response with the production of antibodies, that is performed by the B lymphocytes; and the cell response, that is performed by T lymphocytes.

Viruses are a useful platform for the development of new vaccines. Its protein structure is very stable, and because they are pathogen-associated to molecular patterns, they have a high immunogenic potential. There are several strategies in creating new vaccines: VNPs that are not infectious by chemical modifications, non-infectious VLPs as they lack their genome, and chimeric VNPs[4, 56].

One of the great advantages of the use of VNPs and VLPs as vaccines is the possibility to grow and produce viruses in cell cultures, enabling better control and study of the particles. However, attenuated vaccines are more unstable and more problematic when they are released in the body, and there is a risk that the strain become virulent. The administration of killed viruses would solve this problem, but the immune response is weaker and a higher amount of dose is required, and subunit vaccines are poorly immunogenic. VLPs are considered a type of vaccines that are more stable and safer[56, 57,160].

The two most successful VLP-based vaccines have been licensed to use them in humans. The first vaccine is against *Hepatitis B Virus*. The second, against *Human Papillomavirus*. VLPs of enveloped viruses like *Influenza* or *Human Immunodeficiency Virus* (HIV) have not capacity of replication, but they are problematic in the development of vaccines. For HIV, the VLP vaccine it is not effective, but modifications could improve the strategy[56,58].

The filoviruses *Ebola* and *Marburg* are enveloped viruses that cause lethal and hemorrhagic diseases in humans and non-human primates[80]. Filovirus VLPs are strong immunogens with the potential for development of vaccines. However, the large size and filamentous structure of these viruses has presented a vaccine production difficult. Carra et al. (2015) have prepared nano-VLP of *Ebola virus*, through the sonication of VLPs, and then they purified them by membrane chromatography and filtration, with high stability. In the range of 230 nm of diameter, the particles retain the conformation of the GP proteins of the virus, and the antigenic effectiveness of the vaccine[81].

5.5. The immune response to VNPs and VLPs

Despite all these techniques, VNPs and VLPs have to avoid the immune system, because if the body responds against VNPs and VLPs, efforts are invalid. The toxicity of most viruses being used in Nanobiotechnology applications has not been described. Most of the viruses being developed for these applications are typically not human pathogens. These VNPs from plant cells should not generate an immune response because animal cells do not normally have receptors for the same[9]. There are studies of the oral administration of CPMV particles in mice[82]. The particles can enter into the circulation and disseminate into different tissues. High doses of CMPV showed no apparent toxicity except a lymphoblastic response.

However, the immune response against VNPs and VLPs can be very important. Most of the viruses used in studies of Biomedicine and Nanomedicine are VLPs, and they do not replicate in humans, so the immune response is less important, but the response exists. It has been shown that the structure of a virus capsid is especially immunostimulatory, particularly for B cells, that are cells that produce antibodies[6, 83]. However, there are studies of the use of VNPs that do not generate immune response in vitro; one example is the study of VNPs of *Potato Virus X* (PVX) and *Tomato Bushy Stunt Virus* (TBSV), that are not hemolytic in vitro, not teratogenic and not toxic in a chicken embryo model[84].

One of the most popular approach to reduce the immune response is coating the outer surface of VNPs and VLPs with Polyethylene Glycol (PEG). PEG allows efficient reduction or blocks interactions between nanomaterials and cells of the immune system and proteolytic enzymes, because PEG increases the molecular weight of materials and acts as a shield. PEG is neutrally charged, is highly hydrophilic, non-toxic and is FDA approved. This is an effective strategy to increase water solubility and increase stability, and improves the pharmacokinetics and reduce immunogenicity[59-62].

6*. Detecting viruses*

The detection of viruses becomes ever more important, and it needs effective methods; nanoscale fabrication technologies are increasingly being used to create sensors of biochemical entities. The applications can be very diverse, in the field of clinical diagnosis, bioterrorism and environmental monitoring, among others[85,86].

According to Bhattacharya et al. (2011), detection of viruses in clinical and environmental samples using simple, rapid and inexpensive methods is extremely important. Appropriate control measures can be implemented quickly if the evidence of existence a virus is found[94].

However, the detection of viruses can be too difficult, because sometimes, the concentration of the viruses is not enough. In other cases, the morphology of the particle is unsuitable, or there are inactivators or inhibitors in the solution that contains the viruses[87]. Proteomic analysis allows to know all changes that occur in proteins along all the time that a disease is developed[88], and has contributed to a large degree to the field of clinical diagnosis of diseases[89], but scientists have to make more efforts to develop virus detection methods that could be more sophisticated.

Today, there are many systems to detect viruses, based in nanomaterials or nanodevices. The high ratio of surface to volume of nanomaterials makes them good sensors, and so, they have been developed in the field of Nanotechnology. In addition, the plasmon resonance of nanomaterials have allowed the development of new and different sensors[91].

One example is the detection of the HIV-1, based on the use of *Scanning Tunneling Microscopy* (STM). This virus causes Immunodeficiency Syndrome (AIDS), and it affects the immune system, so different diseases can appear. At the early stage of HIV infections, the symptoms are similar to the flu. If it would exists a system to detect the virus in a early stage of the disease, professionals could prescribe more effective treatments. There are different methods to detect viruses based on Molecular Biology and Immunology, but they can present problems as they are not sensitive enough, and they are time-consuming[90-92].

STM is very useful due to its high-resolution Imaging properties. In accordance with Lee et al. (2015), when a voltage is applied between a sharp metal and an electrically conductive material that is positioned approximately less than 10 nm away, a very small tunneling current is produced. According to quantum mechanics, this electrical current will pass through the surroundings without physical contact between the probe tip and surface. As the tip scans the conducting surface, a proportional change happens in the tunneling current with different density states[91].

In this way, Lee et al. (2015) have demonstrated a system based in STM as a vertically HIV virus sensor[93]. They use a gold substrate with antibodies that bind VLPs of HIV, and gold nanoparticles that have antibodies to bind HIV VLPs. So, the nanoparticles are localized due to immunorreactions, and when the tip scans the sample, and there are VLPs linked to the gold nanoparticles and to the substrate, there is an abrupt change in the tunneling current. The presence and the amount of viruses can be quantified. This system can be extended to detect different types of viruses.

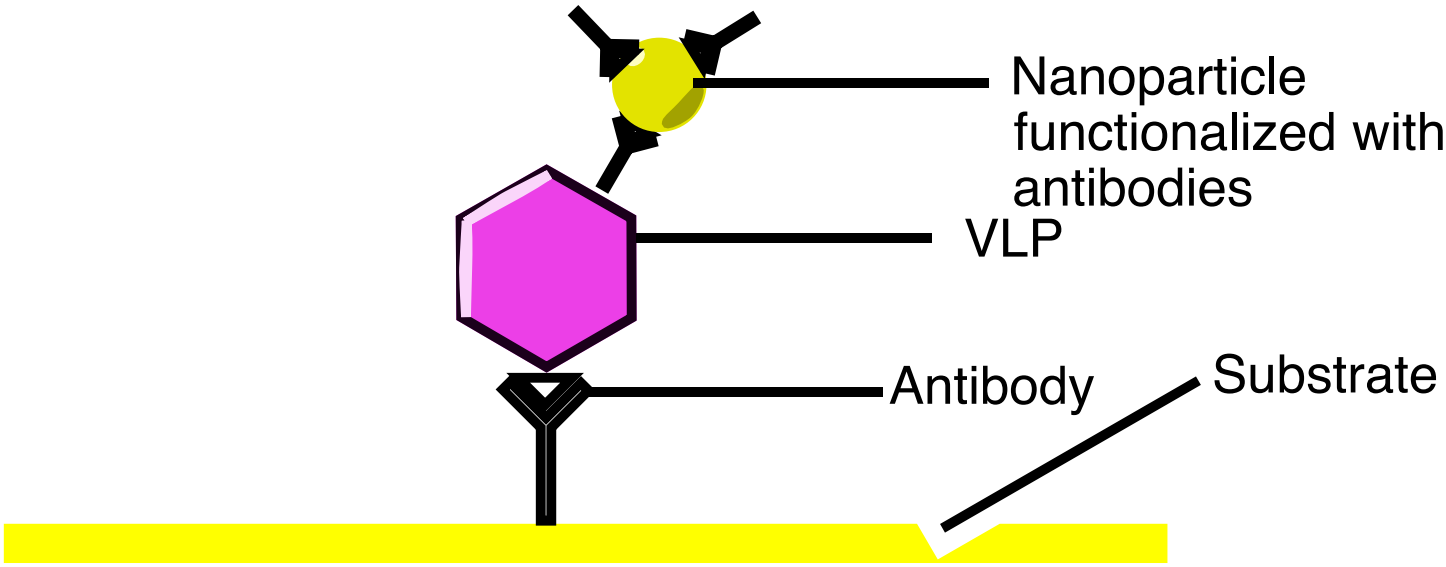

Figure 8: Nanoparticles-antibodies-VLPs complex fixed in a substrate surface.

Meanwhile, carbon nanotubes (CNTs) are interesting nanomaterials that have been developed for biosensing applications[95-98]. Carbon nanotubes have a large surface per unit mass (50-500m^2/g), and good mechanical and electrical properties, so, they can be used in the detection of viral entities. Moreover, CNTs can be functionalized with different materials that bind to different molecules, and these materials can enhance the biocompatibility of the CNTs[99]. Therefore, antibodies can be bound to CNTs, and when a virus binds to antibodies, the immunoaffinity reaction can be detected by a change in the mechanical or electrical properties of the CNTs, like changes in potentiometry, amperometry, voltammetry, colourmetry, or capacitance measurements.The use of CNTs as immunosensors is only an example[94,100,101].

CNTs can be used to detect different viruses across their components. Silva et al. (2014) used CNTs for the detection of the *Dengue Virus*[102]. *Dengue virus* generates a disease that causes epidemics in many tropical and subtropical regions of the world, through of D*engue Hemorrhagic Fever* (DHF) and *Dengue Shock Syndrome* (DSS)[103]. Recently, the non-structural 1 (NS1) protein of *Dengue virus* has been proposed as a marker of DHF, when there are problems with another techniques of detection, like Reverse Transcription PCR (RT-PCR)[104].

Functionalized CNTs can be deposited on carbon electrodes to detect different changes when a certain molecule is linked to functionalized CNTs. However, these processes can be difficult because sometimes the interactions between the CNTs and the electrode surface are weak[105]. In their work, Silva et al. use an amine polymer film that is assembled on carbon nanotubes surface in order to ensure the CNTs are retained. Poly(allylamine) is a molecule that act as a bifunctional linker towards carboxylated carbon nanotubes on one side, and anti-NS1antibodies through their Fc portions on the other side. Glycine is added in order to block the non-specific bindings. So, it is possible to detect the NS1 presence in serum samples, and in

consequence, the presence of *Dengue Virus.* Also, different materials can act as sensors being immobilized in substrates[106].

Different systems are high frequency nanoelectromechanical systems. Ilic et al. (2004) have design silicon cantilevers that are functionalized with antibodies, to measure binding events of *Baculovirus*[107]. When the virus is bound to the specific antibodies that are localized in the cantilever, the total mass of the mechanical oscillator is altered, changing its natural resonant frequency, and this can be registered. This system can be extended to detect different viruses.

7. Nanomaterials against viral diseases

The use of nanomaterials against viral diseases is a good alternative to conventional methods.

Influenza, caused by *Influenza Virus*, is a group of viral respiratory infections that affects the human beings. The mutation of its antigen is so frequent, and it causes pandemic disasters in the world every so often, especially for type A, so pharmacological and pathological researches are very important to combat this virus[108,109].

Fullerenes are the third molecular form more stable of the carbon. They have the form of spheres, ellipsoids or cylinders, and they are constituted by rings of pentagons and hexagons of atoms of carbon[140]. Pursuant to Mroz et al. (2007), the condensed aromatic rings present in the compound lead to an extended p-conjugated system of molecular orbitals and therefore to significant absorption of visible light, making fullerenes good materials for their use in Photodynamic Therapy[110]. Ji et al. (2008) have studied the anti-*Influenza Virus H1N1* activity of fullerene liposomes in Madin-Darby Canine Kidney cells (MDCK), observing the cytotoxicities and its activity rendered by different intensities of lightness with various periods of time[108]. The liposomes are spheric membranes constituted by phospholipids, that can be integrated into cell membranes and serve as a drug carrier. They observed that fullerene liposomes had a significant activity killing the *Influenza Virus*, while the activities in antiadsorption and antireplication were not obvious.

It has been confirmed that fullerenes and its derivatives have strong antiviral activity. Fullerenes solution can inactivate *Influenza Virus* in the allantoic fluid of chicken embryo damaging the viral envelope, and this might be due to the capacity of passing electrons through lipid double membrane[108,111]. Futhermore, Arbogast et al. (1991) have reported that fullerenes produce high quantum of singlet oxygen, that is stimulated by light in the presence of oxygen, and the singlet oxygen kill the viruses by oxidative stress[112].

Herpes Virus Simple type 1 (HSV-1), is one of the most common viral infections in humans and produces different pathologies[113]. The drug acyclovir is used against *Herpes Virus* infections, which is a synthetic analogue of 2-deoxiguanosine[114]. Acyclovir inhibits the replication of the virus. However, there are some problems with this drug, like the low oral absorption[115]. Niosomes are non-ionic surfactant vesicles that form a bilayer structure, that are similar to liposomes, so they can serve as drug carriers[116,117]. Niosomes are preferred compared to traditional liposomes because they have more chemical and physical stability, low toxicity and cost[118,163].

Monavari et al. (2014) have prepared nano-niosomes with the drug acyclovir, and they have studied the effect of them against HSV-1 in cell cultures of HeLa cell lines[119]. The results indicated that the acyclovir loaded nano-niosomes had a significant higher antiviral activity, compared to the free drug. The results suggest that niosomal formulation could be a promising Drug Delivery system for acyclovir.

Ensign et al. (2012) have developed poly(lactic-co-glycolic acid) nanoparticles with PEG, named as mucus-penetrating particles (MPPs), as a vaginal Drug Delivery system against HSV-2[129]. These nanoparticles moved through mucus very quickly, and allow the release of the drug over time, in experiments with mice. Then, they developed MPPs linked to acyclovir monophosphate, and a significant number of mice where protected in comparison to the application of the soluble drug.

However, the emergence of virus strains that are resistant to the drug acyclovir by mutations in the virus, the ability of this virus of have latency in cells, and the adverse effects of another anti-herpetic compounds are a stimulus for the search of new antiviral agents that block the viral entry, between the different processes of the viral cycle[120,121].

Zinc oxide (ZnO) has antibacterial and antifungal properties. Moreover, ZnO nanoparticles have the potential to develop anticancer agents, destroying tumor cells with a high selectivity[122]. Also, ZnO Nanoparticles are widely used in consumer products like in the formulation of sunscreens[123].

Heparan-sulfate (HS) is a polysaccharide of the cell surface, that is involved in viral pathogenesis because it acts as a receptor of the HSV-1. In this case, the use of ZnO nanoparticles could act as an antiviral therapy, by the competition for the HS receptor, and this can be extended to combat different herpesviruses that bind to the cell surface HS[124-126]. In the HS structure there are negative charges, and nanoparticles of ZnO containing filopodia like spikes expand the surface area negatively charged. This concept is used in a study[127] of Mishra et al. (2011). They generated different ZnO micro-nano structures (MNSs), that were capped with multiple nanoscopic spikes, mimicking cell induced filopodia. The nanoparticles compete with HS

by through their partially negatively charged oxygen vacancies on their nanoscopic spikes, affecting viral entry. In the study, they used human corneal fibroblasts, that are a natural target cell for the HSV-1. Under UV-light, more oxygen vacancies were created in ZnO nanoparticles, allowing more viruses to be bound. The major advantage of ZnO nanoparticles is their effectiveness at lower concentrations, the low cost of their synthesis, molecular specificity, and ease to designing nanoparticle capsules coated with additional anti-HSV agents.

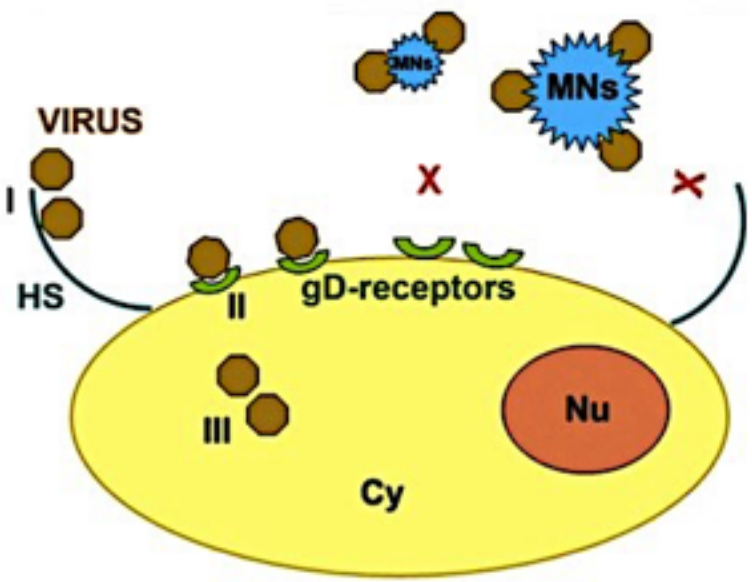

Figure 9: Scheme of the competition between ZnO nanoparticles and the cell receptor HS. Reprinted with permission from ref 127.

Another drug against HSV-1, and HSV-2, are dendrimers. They are polyanionic macromolecules with broad-spectrum antiviral activities and minimal toxicities. Gong et al. (2005) have tested the antiviral action of the SPL7013 dendrimer, and it showed a strong anti-HSV activity[128]. They incubated Vero cells with SPL7013, with an experiment simulating the topical application of drug into the female genital tract. Anti-HSV activity of SPL7013 was not significantly affected by acidic pH or the presence of human serum proteins and human albumin, and they observed the inhibition of virus attachment and entry and inhibition of later stages of HSV replication.

The M*onkeypox Virus* (MPV), is a virus that is similar to the *Variola Virus*, the causative agent of monkeypox in many species of non-human primates and is endemic to central and western África[130]. There is a study[131] realized by James V. Rogers et al. (2008) of the use of silver nanoparticles against *Monkeypox Virus*. They used polysaccharide-coated silver nanoparticles and $AgNO_3$ nanoparticles. Some type of nanoparticles were effective at reducing MPV-induced plaque formation in vitro in cell cultures of Vero cells, in a certain range of concentrations. However, the mechanism by which this inhibition occurs is not known and could involve blockade of host cell binding, disruption of host cell biochemical pathways, or both.

In the case of HIV, two main action mechanisms of action are possible for anti-HIV agents, namely inhibition of HIV-protease or inhibition of HIV-retrotranscriptase[136]. However, this virus is problematic because is a RNA virus, and the retrotranscriptase causes a lot of mutations, so the proteins of the virus change a lot, and the development of efficient drugs is difficult.

The microbicides are a good way to fight against sexually transmitted infections, like HIV. The microbicides include compounds as nonspecific surfactants and detergents. They destroy the envelope of the virus, because they could solubilize the proteins of their structure, among other processes[132].

Dendrimers, molecules that have been mentioned before, are good nanomaterials, because its manufacturing cost is inexpensive, can be easily functionalized, and they are biocompatible. Dendrimers have shown effective anti-HIV-1 activity as non-specific applied microbicides. Also, polymers can act as microbicides[133,134].

The G2-S16 dendrimer is a second generation carbosilane dendrimer scaffold built from a silica core, which is fully capped on the surface with 16 sulfonate groups. Sepúlveda-Crespo et al. (2015) have used the dendrimer G2-S16 to show that the vaginal application of a gel formulation of G2-S16 dendrimer confers protection against HIV-1 in the vagina of humanized mice, without vaginal irritation or lesions, after histological analysis[135].

G2-S16 acts directly on the virus, because it blocks HIV-1 replication at steps prior to the integration of viral DNA into the infected host cell genome. It provides a barrier to infection for long periods, and is effective at non-cytotoxic concentrations in a mice model. Sepúlveda-Crespo et al. (2015) defend that it would be interesting to explore novel routes of administration, like intravaginal rings or locally applied solid films, in order to maximize the activity, safety, adherence, and to test the efficacy of the interaction between condom and microbicide, to study if the properties of the condom could be modified in the presence of the drug.

Fullerene derivatives have been synthesized in order to exploit their potential as inhibitors of HIV aspartic protease enzyme[138]. The union of the fullerene derivatives at this enzyme, causes the alteration of viral processes that are fundamental in the cycle of the virus. In other way, Mashino et al. (2005) reported the HIV-reverse transcriptase inhibition by fullerene derivatives too[137].

Liposomes are useful in the design of intravaginal Drug Delivery systems. Phosphatidylcholine-based liposomes have been prepared by different approaches, and different drugs have been incorporated into them, and in vitro stability have been tested. Liposomes allow prolonged release of the drug in the vaginal tract, and this system can be extended in order to prepare different liposomes with different drugs inside, to fight against different viral diseases[139].

8. Nanomaterials and vaccination

As seen in chapter five, the vaccination is very important to improve the quality of life of animal species, protecting them against different infections. However, the vaccine design is not always useful, because sometimes the vaccine is not active, or needs some type of help to be more immunogenic, o more stable, among others properties. In order to improve this, carriers or adjuvants are used in the development of vaccines. Carriers are strange proteins that bind to an antigen non immunogenic to convert it into an immunogenic antigen. In the other hand, adjuvants are a material that is bound to an antigen to accelerate or enhance the immunogenic response of the vaccine. The difference between carriers and adjuvants are that the adjuvants have a non-stable union to the vaccine[141].

Hepatitis B Virus (HBV) infection is an important public health problem. A lot of therapies have failed in the control of the viral cycle in most patients. DNA vaccines are a interesting strategy for elicit immune responses against HBV infection. DNA vaccines consist on the introduction of DNA in the body, that encodes to an antigenic protein, inducing the activation of cells of the immune system. This allow to induce the humoral and cellular response. However, this type of vaccines present a problem, that is the stability and delivery of the DNA that is not very efficient, because the vaccine is degraded easily. Effective vaccine vectors are desirable that can protect DNA vaccines[142-145].

Layered Double Hydroxide (LDH), are nanomaterials with applications in catalysts, absorption, pharmaceutics, and photochemistry, and they act as a efficient vaccine delivery system for their properties of low cytotoxicity, good biocompatibility and protection of loaded DNA vaccines[146,147,161,162]. In order to enhance the adjuvant immune activity of inorganic vaccine delivery vectors, Jin Wang et al. (2014) have synthesized SiO_2@LDH as a delivery vehicle of a DNA vaccine which encodes *Hepatitis B Virus* surface antigen protein[145].

SiO_2@LDH have a large surface area, so these nanoparticles are good materials for the delivery of a DNA vaccine. Also, the nanoparticles do not show any cytotoxicity and can totally protect DNA from DNases digestion. When SiO_2@LDH nanoparticles internalize in the acidic environment of endosomes and lysosomes in macrophages, the material collapse and the vaccine is released, and the nanoparticles active NF-kB factor that induces the transcription of immune-relevant genes. So, the vaccine is protected, and can activate cells of the immune system.

Porcine Reproductive and Respiratory Syndrome (PRRS) is an important economically disease in pigs, caused by *Porcine Reproductive and Respiratory Virus* (PRRSV)[148]. There is a study[149] where Binjawadagi et al. (2014) have prepared a complex constituted by inactivated/killed PRRSV vaccine antigens and biodegradable (poly/lactic-co-glycolic acid) nanoparticles. Then, they coupled this complex with a potent mucosal adjuvant, the whole cell lysate of *Mycobacterium*

tuberculosis (*M. tb* WCL). The combination of the first complex and unentrapped *M. tb* WCL elicited a strong humoral and cell immune responses in the lungs.

A different disease that affects animals are Foot-and-Mouth Disease (FMD), which causative agent is *Foot-and-Mouth Disease Virus* (FMDV). This virus affects artiodactyls, and is one of the most contagious of all animal diseases[150]. While inactivated FMDV vaccines have been used effectively to control outbreaks, this vaccine has not been useful[151]. Peptide-based vaccines have many advantages, as high stability, standardized production method, and poor infectivity[152].

One way to increase the immune response without adverse effects is to conjugate vaccines to inert nano-beads[153,154]. The humoral and cell immune responses have been studied with the use of FMDV specific peptides, covalently conjugated with nano-beads as an adjuvant in sheep[150]. Carboxylated polystyrene inert nano-beads showed the potential to induce the humoral and cell immune responses, allowing immunogenicity against this disease.

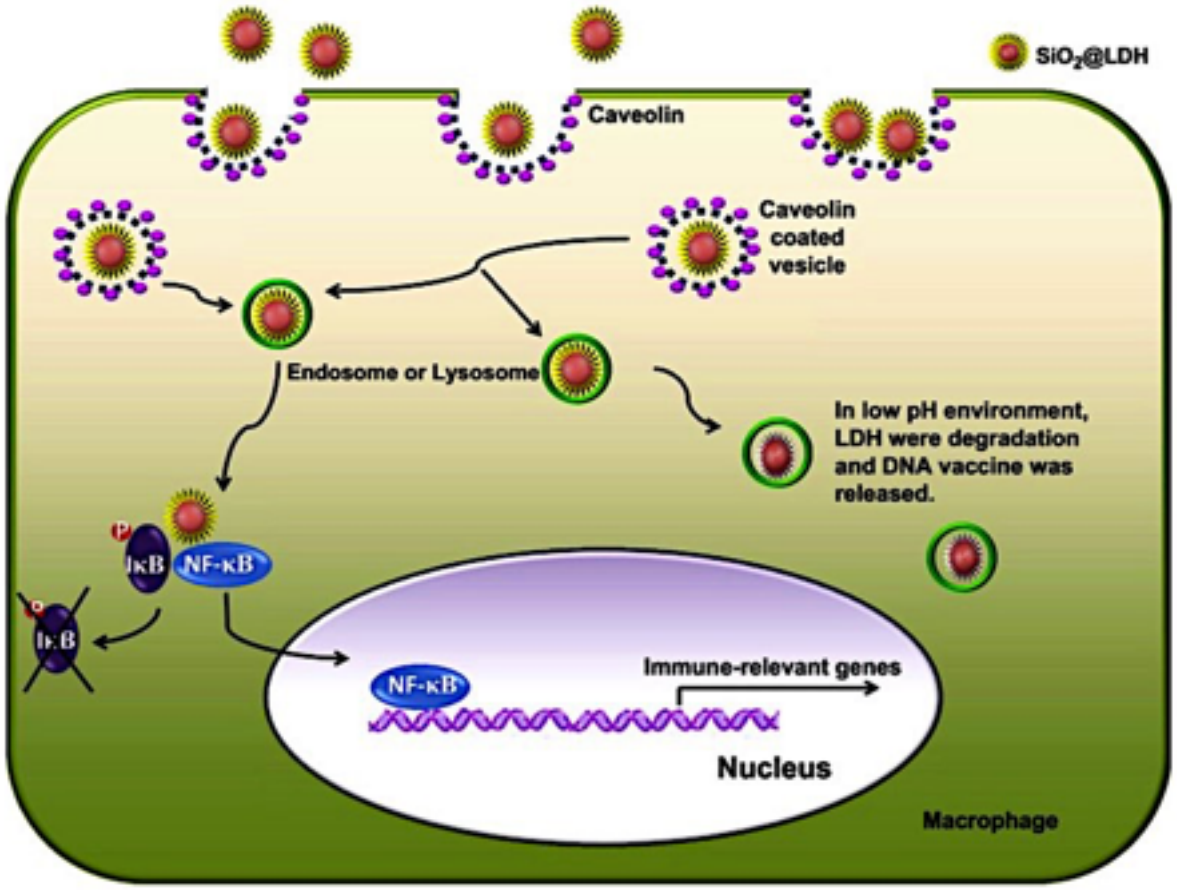

Figure 10: Diagram of the entry of the vaccine via endocytosis, with the release of the vaccine in endosomes and lysosomes, and the activation of immune-relevant genes. Reprinted with permission from ref 145.

Respiratory Syncytial Virus (RSV) is one of the most common causes of viral deaths in infants around the world[155]. Firdous et al. (2014) have studied the use of an osmotically active polysaccharide-based Polysorbitol Transporter (PST) prepared from sorbitol diacrylate and low-molecular-weight Polyethylenimine (PEI) showing a potent and safe adjuvant activity and acting as an effective delivery tool for the RSV glycoprotein antigen[156]. Antigenic polysaccharide-based vaccines could elicit memory B cells and improve the production of antibodies[156,157].

These are just some examples of nanomaterials that can be useful in the development of vaccines in the fight against viral diseases. Everyday new nanomaterials are developed, and new vaccines are created. However, as seen in the chapter of *Nanotechnology applications used in Biomedicine and Nanomedicine*, there is a risk of cause a immune response. It would be desirable that nanomaterials used in the treatment of viral infections and the development of vaccines should not cause an immune response.

10. Conclusion

In this work different fields of Viruses and Nanobiotechnology have been explored, where Biology, Chemistry, Physics, Molecular Biology, Medicine and Materials Science are integrated.

Chemical modifications of the viruses and their studies in the treatment of diseases, like cancer, are a great promise of future, because today, not all the treatments are effective against these diseases. By this manner, it is very interesting the development of new therapies, and viruses are good candidates, for their specificity for certain cells. Chemical modifications of them allow to reduce the immune response of the body and make more effective the therapy. These utilities can be extended in the development of Medical Imaging, Photodynamic Therapy, and VNP and VLP based vaccines.

The development of new sensors of viruses can be very interesting, but we have to keep in mind that today there are already different techniques and systems that can present problems of detection, but however they are very efficient in detecting viral entities, and not so expensive. So, the development of new sensors can be very useful, but in cases that detection of viral entities was complicated, or was non fast as we want. The use of nanomaterials in the fight against viral infections and in the development of new vaccines can be very useful, because every day, viruses suffer mutations and drugs and vaccines could become inefficient. So, it is very important to have new alternatives to fight against viruses that causes global pandemics. However, the new alternatives should be efficient, cheap and useful.

In conclusion, Viral Nanotechnology is an emerging field with infinite possibilities of exploitation in Biomedicine and Nanomedicine, but it is important to search new nanobiomaterials with good characteristics for these achievements.

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