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Virus-based nanoparticles as platform technologies for modern vaccines

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Abstract

Nanoscale engineering is revolutionizing the development of vaccines and immunotherapies. Viruses have played a key role in this field because they can function as prefabricated nanoscaffolds with unique properties that are easy to modify. Viruses are immunogenic through multiple pathways, and antigens displayed naturally or by engineering on the surface can be used to create vaccines against the cognate virus, other pathogens, specific molecules or cellular targets such as tumors. This review focuses on the development of virus-based nanoparticle systems as vaccines indicated for the prevention or treatment of infectious diseases, chronic diseases, cancer, and addiction.

Introduction

Vaccines are designed to elicit a strong immune response and to provide long-lasting protective immunity by generating neutralizing antibodies, activating cellular immunity and inducing immune memory [1, 2]. The earliest reports of vaccination were subjects protected against smallpox by exposure to powders from infected scabs. However, Edward Jenner presented the first formal description of a vaccine in 1798, when he observed that milkmaids previously infected with the less virulent *Cowpox virus* were no longer susceptible to smallpox. In 1967, the World Health Organization (WHO) oversaw a worldwide smallpox eradication program, which was completed by 1980 [3]. Since then, eradication programs have been established for other diseases such as polio, measles, mumps, rubella, and malaria [4, 5].

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Vaccines have also been developed against other prevalent infectious diseases, such as hepatitis B, rabies, anthrax, and cholera [6–9]. The development of vaccines has achieved an immense socioeconomic impact by reducing the burden of erstwhile pandemic diseases responsible for widespread morbidity and mortality. Even so, several major pathogens cannot yet be controlled by vaccines including *Human immunodeficiency virus* (HIV) and hemorrhagic fever viruses, such as those responsible for the recent Ebola outbreak affecting West African countries. More recently vaccines have also been against non-infectious diseases including cancer and chronic disease.

Virus-based nanoparticles as platform technologies

Nanoparticle-based vaccines have been developed using a diverse range of materials (Figure 1), including synthetic particles (e.g. gold, polymers or lipid micelles) and biological particles (e.g. nucleic acids and proteins, including viruses) [10, 11]. We consider two broad types of particles in the latter category: virus-based nanoparticles (VNPs) that feature a modified capsid encapsulating the virus genome, and virus-like nanoparticles (VLPs) that comprise protein components alone.

Virus-based materials have many beneficial properties. Their proteinaceous, highly-ordered, multivalent structures, when combined with an appropriate immune adjuvant, often elicit robust cellular and humoral immune responses [12]. They display antigens in a repetitive array (which promotes B cell crosslinking and subsequent activation) and pathogen-associated molecular patterns (PAMPs) that induce stronger and longer-lasting antigen-specific immune responses than soluble antigens [13–15]. The single-stranded viral RNA (ssRNA) found in VNPs is also a PAMP, and this is a natural ligand for Toll-like receptors 7 and 8 that induce cytokine expression [1617–19]. The size range of virus particles (20–500 nm) means they are efficiently taken up by antigen presenting cells (APCs), including dendritic cells (DCs) and other phagocytes, thus stimulating T cells [20, 21].

Viral vaccines can be divided into four categories (Figure 2): live-attenuated, inactivated, subunit vaccines, and native or recombinant VNP/VLP structures. The latter are considered safer because there is no risk of virulence, yet stronger than inactivated viruses or subunit vaccines because they induce a robust immune response without multiple doses [22, 23]. Native VLPs lack the viral genome but are otherwise identical to the infectious virus, making them highly immunogenic but unable to replicate. These are particularly suitable when the native virus replicates and causes disease in humans. VNPs retain the genome and are therefore easier to produce by relying on natural virus replication. This format is particularly suitable when the native virus does not replicate in humans, i.e. bacteriophage and plant viruses. Recombinant VLP/VNP formats add an important further layer of advantages because they can be engineered to present antigenic epitopes of a counterpart virus or any other disease-associated antigen. VLPs and VNPs can be manufactured in heterologous production systems, including plants, mammalian cells, yeast and bacteria [24].

Chemical and genetic engineering of virus-based scaffolds

Viruses comprise many identical copies of one or more coat proteins arranged in helical or icosahedral symmetry to form a capsid that encapsulates the genome. The structure of many virus capsids has been solved at atomic resolution, allowing site-specific modification and the multivalent display of antigenic epitopes on particular surface loops or the N/C-terminal region of the coat protein. Epitopes and/or other immunostimulatory molecules can be introduced by chemical engineering (bioconjugation) of particular residues (Figure 3) or genetic engineering of the coat protein sequence (Figure 4).

Chemical conjugation strategies

Antigenic peptide sequences can be added to a virus coat protein by chemical modification strategies that target five of the 20 naturally occurring amino acids: lysine (amine functional group), glutamic and aspartic acid (carboxylate functional group), cysteine (thiol functional group), and tyrosine (hydroxyl functional group). Lysine residues contain a highly nucleophilic amine that reacts with isothiocyanate or *N*-hydroxysuccinimide (NHS) esters. Amines can also be covalently attached to carboxylate groups through formation of an amide (peptide) bond, facilitated by a carboxylate-selective coupling agent such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). Glutamic and aspartic acid residues contain carboxylate groups that can be modified using EDC to react with amine-functionalized peptides resulting in the formation of a stable amide bond, in the mirror image of the reaction described above. Cysteine residues contain thiol groups that can be reacted with haloacetyls or maleimides. Finally, tyrosine residues contain a phenolic hydroxyl group, which can be modified using diazonium coupling strategies, although this is more complex than the other reactions listed above.

As well as these direct bioconjugation strategies, bifunctional linkers can be used to introduce additional functionalities that are not naturally found in virus coat proteins. Bio-orthogonal reactions, including ‘click chemistries’ such as Cu(I)-catalyzed azide-alkyne cycloaddition are particularly useful because the kinetics of the reaction are much more efficient than standard coupling. Ligation handles can be introduced via the bioconjugation of an azide or alkyne-NHS ester to a lysine side chain, or through the incorporation of non-natural amino acids *in vitro*. The diverse chemistries used to engineer viruses have been reviewed in detail [25].

Genetic engineering strategies

Unlike synthetic nanoparticles, VNPs can be modified not only chemically but also genetically, i.e. the nucleic acid sequence encoding the coat protein can be changed to exchange particular amino acids or introduce additional contiguous amino acids to form linear epitopes. Three major approaches are used to insert additional peptides into the virus coat protein, resulting in a coat protein fusion or chimera: direct fusion, linker fusion, and the “protein overcoat” strategy. In the direct fusion approach, the foreign peptide is linked directly to the N-terminus [26–28] or C-terminus [28–31] of the coat protein or inserted into flexible surface loops presented on the capsid surface [29, 32]. Although the external surface is usually chosen for presentation of native antigens recognized by B cells, the internal surface

may be more suitable in some applications that involve the presentation of processed peptides [33, 34]. In contrast, linker fusion involves the inclusion of a short sequence of amino acids (e.g. multiple glycine residues) between the foreign peptide and the end of the coat protein to allow flexibility. Finally, the "protein overcoat" strategy places the *Foot and mouth disease virus* (FMDV) 2A sequence between the foreign peptide and coat protein sequences, causing an inconsistent ribosomal skip during translation. The outcome is a mixture of native coat proteins and fusion proteins, which is useful if the inserted sequence is so large that its presence on every copy of the coat protein would prevent virus assembly [35].

VNP and VLP vaccines and immunotherapies

The first vaccines were developed against infectious diseases and likewise the first VLP and VNP vaccines were developed as strategies to contain the disease caused by the corresponding native form of the virus. However, as chemical and genetic engineering strategies have become more sophisticated, VLPs and VNPs have been adapted into platform technologies for the presentation of more diverse antigens, including abnormal self-proteins that can be used to treat chronic diseases and cancer. Several key vaccines based on VLPs or chimeric VNPs are summarized in Table 1.

Infectious diseases

HIV—HIV (Figure 5) is unusual in that it primarily attacks the immune system and therefore destroys the very cells whose function is to neutralize it. By disabling the immune system, HIV not only achieves a successful infection but it also renders the body susceptible to other adventitious pathogens, i.e. acquired immunodeficiency syndrome (AIDS). There is no cure for HIV/AIDS. More than 35 million people are currently infected with HIV, two thirds of the infected population living in sub-Saharan Africa [92]. The current best treatment option is highly active antiretroviral therapy, a cocktail of drugs consisting of a non-nucleoside reverse transcriptase inhibitor and two nucleoside analog reverse transcriptase inhibitors [93]. Early HIV vaccine candidates based on inactivated or attenuated viruses were ineffective or unsafe [94, 95]. More recent vaccine development strategies have focused on eliciting both humoral and cell-mediated responses targeting HIV envelope proteins.

Recombinant VLPs and VNPs displaying full-length HIV envelope proteins, individual glycoproteins, glycoprotein precursors or fragments thereof, on the surface of carriers such as *Flock House virus* (FHV) [96], *Hepatitis B virus* (HBV) [97], papillomaviruses [37], bacteriophages Qβ, AP205 [36] and MS2 [98], and plant viruses such as *Tobacco mosaic virus* (TMV) [99, 100] and *Potato virus X* (PVX) [27]. The membrane-proximal external region (MPER) of gp41 can be recognized and neutralized by monoclonal antibodies thus providing a good target for a vaccine candidate [36]. Accordingly, a set of gp41 peptides chemically conjugated to VLPs derived from phage AP205 elicited high-titer, peptide-specific antibodies in mice. Depending on the peptide, sera were able to neutralize a highly-sensitive laboratory strain of HIV-1 and a less-sensitive primary isolate, but not a clade C primary isolate. Some sera exhibited antibody-dependent cell-mediated cytotoxicity (ADCC) in infected cells, indicating that ADCC epitopes are most likely located in the distal

region of gp41 [36]. The highly conserved epitope of gp41 (ELDKWA) has been genetically fused to the N-terminus of PVX, among other platforms [27, 101, 102]. Sera from mice immunized with these chimeric virus particles contained high IgG titers specific for HIV-1 MN gp160-derived synthetic peptide (H66), and were able to neutralize HIV-1. Additionally, human DCs pulsed with the vaccine triggered the proliferation of peripheral blood lymphocytes *in vivo* [27]. Multiple gp41 epitopes such as a trimeric recombinant gp41 (rgp41), which contains several conserved gp41epitopes, were conjugated to influenza virosomes. Vaccinated rhesus monkeys were challenged intravaginally 13 times with a heterologous simian HIV (SHIV). All subjects vaccinated through intramuscular or intranasal route were protected from challenge, whereas only 50% of the intramuscular-only group was protected [45].

Other HIV vaccine development approaches include the targeting of host cell receptors such as the C-C chemokine receptor CCR5 co-receptor used by macrophage strains of HIV-1 [103–107]. Bovine papillomavirus type 1 (BPV-1) VLPs were engineered to express CCR5 peptides. Vaccinated mice produced high antibody titers against CCR5, and functional studies demonstrated that sera displaced the native CCR5 ligand in a competition assay. Most importantly, sera from immunized mice neutralized HIV-1 in cells transfected with a human-mouse chimeric receptor of CCR5 [37]. CCR5 peptides have also been conjugated to bacteriophage Qβ. Two peptides, representing the N-terminus (EC1) or second extracellular loop (ECL2) of macaque CCR5 (mCCR5), were conjugated to Qβ and administered to rhesus macaques. Animals immunized with Qβ-EC1 and Qβ-ECL2 produced high titers of anti-CCR5 antibodies. When vaccinated animals were challenged with SIV, the viral load was lower than in non-vaccinated controls [38].

Another promising strategy is the combination of ALVAC-HIV, a vaccine based on *Canarypox virus* (vCP1521), and AIDSVAX (VaxGen), which consists of gp120 from two different HIV strains [39, 43]. Unlike the vaccines discussed above, the ALVAC *Canarypox virus* vector contains the HIV *env*, *gag*, and *pol* genes [40]. The two treatments were tested together in a clinical trial in Thailand (RV 144). Vaccinated subjects showed a 31% lower rate of HIV infection compared to the placebo group [43]. ALVAC-HIV was also tested alone in a pediatric HIV trial in Africa, involving infants born to HIV-positive mothers. Low levels of binding antibodies were detected in one subject as expected because the subjects did not receive a gp120 boost [40]. The addition of recombinant glycoprotein subunit vaccine (rgp120) to ALVAC-HIV resulted in higher levels of specific-binding serum antibodies in infants that were distinguishable from maternal antibodies. Additionally, 50% of subjects who received both ALVAC-HIV and rgp120 generated neutralizing antibodies against homologous strains of HIV [41].

Ebola virus disease and related diseases—Ebola virus disease is caused by four different filoviruses of the genus *Ebolavirus*: *Bundibugyo ebolavirus*, *Sudan ebolavirus*, *Tai Forest ebolavirus*, and the eponymous *Ebola virus* (formerly *Zaire ebolavirus*) which is the most dangerous and prolific (Figure 6). The ease of infection [108, 109] and lack of clinically approved treatment produces mortality rates of up to 90% [110].

VLP/VNP vaccines against *Ebola virus* are currently in the development pipeline, using either complete Ebola VLPs or specific components such as the viral matrix protein (VP40), nucleoprotein (NP) and glycoprotein (GP) displayed on other viruses. Recombinant VLPs containing *Ebola virus* VP40 and GP were constructed using a baculovirus system. The system yielded high levels of GP-specific antibodies in mice, particularly the IgG2a subtype which is needed to achieve protective immunity. Additionally, VLPs induced the secretion of IL-6, IL-10, IL-12, and TNFα from DCs, confirming their adjuvant and immunostimulatory properties. Serum from vaccinated mice was also able to block the infection of JC53 cells by a pseudotyped virus [47]. The immunization of rodents with VLPs comprising the viral envelope (including GP, NP, and VP40) has also conferred protection against Ebola challenge [48]. Furthermore, immunized cynomolgus macaques were completely protected when challenged with *Ebola virus* [48]. VLPs based on recombinant *Vesicular stomatitis virus* (rVSV) expressing *Ebola virus* glycoproteins were shown to protect mice and non-human primates against a lethal challenge with homologous *Ebola virus* after a single injection [111, 112]. Post-exposure protection was also conferred [113]. The rVSV-EBOV vaccine showed a good safety profile in non-human primates and pigs [114, 115] and was able to prevent the disease when administered during an outbreak in a ring vaccination strategy [50].

Other examples include the application of an inactivated *Rabies virus* fused to *Ebola virus* GP (INAC-RV-GP) resulting in a strong, multivalent humoral response against both viruses in mice and non-human primates, protecting the animals against both diseases [51, 116]. The titer of neutralizing antibodies was increased further by the addition of an adjuvant and resulted in 100% protection from a lethal challenge [52]. The inactivated *Rabies virus* platform has also been expanded to express the GP from other filoviruses, including *Sudan ebolavirus* and Marburg virus [52].

Influenzavirus—Seasonal influenza epidemics cause up to 500,000 deaths every year [117–119] as well as regular pandemics, which can result in millions of deaths in a relatively short time span [118, 120]. Seasonal influenza epidemics are typically caused by human influenzaviruses (Figure 7) that have acquired mutations, whereas pandemics occur when the influenzaviruses cross species barriers [121, 122]. Seasonal vaccines are typically based on whole-inactivated viruses or live-attenuated viruses, both of which achieve good protection and significantly improve public health [123]. However, these vaccines are based on hemagglutinin (HA) and neuraminidase (NA), the major targets of the immune system [123]. Epitopes on both proteins are highly prone to genetic drift, leading to the rapid emergence of influenza strains that are not genetically similar to the strains covered by the vaccine [53, 124–126]. Seasonal vaccines must therefore be updated every year, and vaccines against pandemic strains must be developed in response to the pathogen, a retroactive strategy that places millions of people at risk. Preclinical and clinical research has focused on the development of a proactive universal influenza vaccine based on more conserved epitopes such as the matrix proteins (M1 and M2) and nucleoprotein (NP), which undergo drift more slowly [127, 128]. Other strategies include the development of multivalent VLPs with HA and/or NA epitopes from diverse strains, combined with immunostimulatory molecules.

Recombinant influenzavirus VLPs based on HA, NA, and M1 have recently been used to develop heterotypic vaccines [53–55, 57]. Studies in mice and ferrets indicate that H1N1 VLP

vaccination protects against challenges with the homologous subtype (H1N1) as well as a heterologous subtype (H5N1), and intranasal administration elicited higher IgG and IgA titers than intramuscular vaccination [53]. These VLP vaccines were well-tolerated in a phase II study [55]. Other VLPs have been developed based on the highly pathogenic avian influenza (HPAI) H5N1 and avian-origin influenza A (H7N9) [54, 57]. The H5N1 VLP was composed of HA, NA, and M1 from H5N1, whereas the H7N9 vaccine was composed of HA and NA from H7N9 and M1 from H5N1 [54, 57]. Mice vaccinated with H5N1 VLPs were challenged with homologous or heterologous (H5N8) strains and all survived. Ferrets immunized with H7N9 VLPs plus adjuvant produced high titers of H7N9-specific neutralizing antibodies, and viral loads in the lungs and viral shedding were both reduced compared to controls [54].

Influenzavirus epitopes have also been expressed on the surface of VLPs based on HPV core protein (HBc) [58], *Infectious bursal disease virus* (IBDV) [61], PVX [63], *Papaya mosaic virus* (PapMV) [60, 62], Adenovirus[59], and *Simian virus 40* (SV40) [129]. Several of these platforms were used to display HA, NA, and matrix proteins, similar to the influenzavirus VLPs.

Influenza vaccines can also be developed to promote the formation of inducible bronchus-associated lymphoid tissue (iBALT), which plays a role in adaptive immunity in the lungs, similar to the role of the spleen in primary adaptive immunity [130]. Small heat shock protein (sHSP) cages are structurally similar to VLPs, and when administered to the lung they promote the formation of iBALT, which includes B cells, follicular DCs, and $CD4^+$ and $CD8^+$ T cells. Mice treated with sHSP cages were protected from primary influenzavirus infection, as well as from a secondary infection from a different strain of the virus [65]. The sHSP cages also induced antibody class-switching when mice were challenged with influenzavirus, increasing the amount of IgA and IgG present in the lung [64].

Seasonal influenza vaccines are usually produced in eggs, or in avian or mammalian cell cultures. However, pandemic strains that develop when viruses cross the species barrier (particularly the avian-to-human barrier) are more difficult to produce in avian cells and alternative production platforms are required. One particularly attractive option is to produce the influenzavirus VLPs in plants. The H5 and H1 proteins have each been successfully expressed in *Nicotiana benthamiana* plants and can self-assemble into VLPs. The molecular farming of VLPs has several advantages: in planta production greatly reduces the risks associated with human viruses since plants do not support the replication of human viruses; and the process is highly scalable. For example, Figure 8 shows the Medicago production facility. Mice immunized with plant-derived H5-VLPs were protected from homologous and heterologous viral challenge [131]. Furthermore, when the plant-derived VLPs were tested in a phase I clinical trial, none of the subjects developed allergy or hypersensitivity symptoms, and the IgG and IgE responses to plant-derived epitopes returned to baseline after 6 months. Additionally, no IgE response was observed in response to the glycan motif, MMXF, which is associated with allergenicity [56], demonstrating a good safety profile of the vaccine.

Cancer

Cancer is one of the leading causes of death worldwide, with 14 million new cases diagnosed every year and over 8 million cancer-related deaths. Although cancer includes a diverse spectrum of diseases with different causes, sites of origin, and clinical outcomes,

they are all defined by six hallmarks: sustained proliferative signaling, evasion of growth suppressors, promotion of invasion and metastasis, limitless replicative potential, induction of angiogenesis, and resistance to programmed cell death[132]. Some cancers are caused by viral infections and can thus be prevented with vaccines. The first VLP vaccine offering protection against a cancer-causing virus (*Hepatitis B virus*, HBV) was approved in 1981 for infants. Two vaccines against *Human papillomavirus* (HPV) have been approved more recently for the prevention of cervical cancer, or oropharyngeal cancers. In addition to these FDA-approved vaccines, numerous VLP vaccine candidates are being investigated for the prevention or treatment of lymphoma, leukemia, melanoma, and breast cancer.

VLP-based cancer vaccines can also be developed to enhance the tumor-antigen specific T-cell response and elicit antibodies against tumor-specific surface antigens. In addition to papillomaviruses [70], such vaccines have been developed using bacteriophages [67] and plant viruses [66, 68, 69] as delivery platforms because the native viruses do not infect or replicate in human cells so the virus genome can be left intact. The coat proteins of TMV and bacteriophage Qβ have been modified to present the tumor-associated carbohydrate antigen (TACA), which normally has low immunogenicity. TMV-TACA generated much higher titers of antigen-specific antibodies than the soluble form of the antigen, whereas Qβ-TACA elicited a stronger humoral response to the TACA than the soluble form or TACA attached to other nanoparticles [67]. The resulting IgG antibodies also reacted strongly *in vitro* against cells expressing the antigen [67].

Tolerance against self-peptides with low immunogenicity can be broken using VLP/VNP-based immunotherapy platforms. For example, the immunogenicity of melanoma T-cell epitopes p15e and tyrosinase-related protein-2 (Trp2) can be increased by presenting them together on a single bivalent TMV particle, improving cellular immunity and conferring protection against tumor challenge [68]. PVX has been modified with the idiotypic (Id) immunoglobulin from B-cell lymphomas, a weak tumor antigen. When administered to mice, Id-PVX induced high titers of anti-Id antibodies, which prolonged survival after lymphoma challenge [69].

The FDA-approved vaccines for HPV are based on papillomavirus VLPs. However, these VLPs can also be modified to express other tumor antigens, such as human mucin-1 (MUC-1), which is a marker of ductal adenocarcinoma. BPV-1 particles modified with a MUC-1 epitope were administered to mice, which were later challenged with a MUC-1^+ lymphoma cell line. T cells were strongly induced in the vaccinated mice and their tumors grew more slowly, resulting in a smaller tumor mass at the end of the study [70]. We will consider HPV vaccines and hepatocellular carcinoma vaccines in more detail in the following sections because commercial vaccines are already available. We will also discuss HER-2^+ breast cancer vaccines.

Prophylactic vaccines to protect against cervical cancer caused by HPV— Cervical cancer is the fourth most common cancer in women and more than 500,000 new cases are diagnosed each year [133, 134]. HPV, usually sexually transmitted, is implicated in 90% of cervical cancers [135, 136]. Among more than 150 known strains of HPV, up to 20 are designated high risk because they cause almost all cervical cancers [137]. The two highest-risk

strains are HPV-16 and HPV-18, which are responsible for 70% of all cases [138, 139]. There are currently two FDA-approved prophylactic vaccines for HPV: a bivalent vaccine (Cervarix) that protects against strains 16 and 18, and a quadrivalent vaccine (Gardasil) that protects against strains 6, 11, 16, and 18. Both vaccines are based on VLPs composed of the HPV L1 coat protein (Figure 9) [71] combined with adjuvants to further boost the immune system. Both vaccines have proven efficacious after a three-dose schedule. However, the L1 protein is not conserved across all serotypes, so these vaccines only offer protection against the specific serotypes within each formulation [140–142].

The development of VLPs based on the more conserved L2 coat protein would offer increased cross-protection against multiple serotypes, and the next generation of HPV vaccines is likely to be based on this principle [72]. L2 is naturally shielded from the immune system [143] but vaccination with L2 can nevertheless protect against a range of HPV serotypes [144–146]. The first vaccines based on L2 were limited in efficacy by low antibody titers and the need to sufficiently protect against all high-risk serotypes [147, 148] so VLPs have been considered as a strategy to overcome these limitations.

Bacteriophage MS2 has been used to express L2 epitopes from HPV strains 16 and 31, individually or as a bivalent formulation. MS2-16L2 was previously shown to confer protection against 11 HPV serotypes but not HPV31 [71]. Mice immunized with the individual constructs (MS2-16L2 or MS2-31L2) were protected against some strains, whereas the bivalent formulation (MS2-16/31L2) elicited high antibody titers across a panel of HPV serotypes and strongly neutralized all HPV pseudoviruses [72]. In a complementary approach, bacteriophage PP7 was modified to express L2 from HPV strains 16 and 18 (which are closely related) and 1 (which is more distant) individually and in pairwise combinations (PP7-18L2, PP7-18/1L2 and PP7-16/18L2). Mice immunized with PP7-18/1L2 only produced antibodies against HPV1 peptides, whereas PPV-18/16L2 elicited antibodies that bound strongly to HPV16 and HPV18, as well as HPV1, HPV5, and HPV6. Only mice vaccinated with PPV-18/16L2 were able to neutralize an HPV-6 pseudovirus, a heterologous serotype [72].

The high-risk HPV strains 18, 45, and 59 have been targeted by inserting a cross-neutralizing epitope from HPV45 L2 into a surface loop of HPV18 L1 and creating VLPs from the chimeric construct (18L1-45RG1). L2-specific antibodies from vaccinated rabbits reacted against HPV strains 39, 45, 68, and 70 (which are members of the same clade as HPV45 and HPV18). Additionally, when mice were passively immunized with immune sera from rabbits, they were protected against a challenge with HPV strains 18, 39, 45, and 68[73].

Vaccines to treat cancers caused by HPV—Although prophylactic vaccines have been successful, they are unable to treat established tumors. The development of HPV therapeutic vaccines has focused on the E6 and E7 oncoproteins, which are necessary for tumor development and are expressed in all cervical cancer cells [149]. For example, HPV16L1 was genetically modified to express the HPV16 E7 protein. In mice, these recombinant VLPs induced L1-specific antibodies, as well as cytotoxic T cells that recognized L1 and E7 [150–153]. In a phase I trial, patients with proven ectocervical CIN 2/3 lesions who were also HPV16 mono-infected, were treated with HPV16L1E7 VLPs.

Approximately 50% of vaccinated patients exhibited a 50% reduction in lesion size following the final vaccination [74]. Further improvements of this therapeutic strategy include the incorporation of T-cell epitopes from HPV16 E6 and E7. Preclinical studies in mice showed an 85% reduction in tumor size when immunized with such recombinant VLPs [75].

Vaccines targeting hepatocellular carcinoma caused by HBV—Liver cancer causes 600,000 deaths per year and there are 700,000 new cases, approximately 500,000 of whom are male, making it the fourth most common form of cancer in men [133]. Among these cases, 95% are classified as hepatocellular carcinoma [154], which is associated with risk factors such as alcoholism, hepatitis B, hepatitis C, and liver cirrhosis [155, 156]. HBV (Figure 10) is responsible for about 50% of all primary hepatocellular carcinomas. A vaccine against HBV has been available since 1981, and is on the World Health Organization's List of Essential Medicines. This vaccine is a VLP comprising the HBV surface antigen (HBsAg), which is administered as two or three injections within one year. The vaccine provides lasting immunity against HBV by producing anti-HBV antibodies [157, 158].

The prophylactic HBV vaccine has greatly reduced the incidence of hepatocellular carcinoma but a therapeutic vaccine is needed to treat established disease. One of the key targets is the HBV X protein (HBx), a regulatory protein that promotes carcinogenesis and is expressed at high levels in hepatocellular carcinoma [76, 159]. The HBc has therefore been genetically modified to express dominant HBx-derived cytotoxic T-cell epitopes, as well as the universal Th-cell epitope, pan-HLA DR-binding epitope (PADRE). These chimeric proteins self-assemble into VLPs, and mice vaccinated with this formulation inhibited tumor growth up to 30 days after tumor challenge, while also eliciting a strong T-cell response [76].

Vaccines targeting HER-2$^+$ breast cancer—Breast cancer is the most common form of cancer in women, as well as a minor form of cancer in men, with over one million cases diagnosed each year [133]. There are five molecular subtypes of breast cancer: normal-like, luminal A, luminal B, HER-2$^+$, and triple negative [160]. Each is defined by the expression of different receptors and the prognosis varies accordingly. HER-2$^+$ tumors overexpress the HER-2/neu/ERBb2 receptor. They do not express hormone receptors and they are associated with aggressive tumors, high rates of metastasis, and an overall poor prognosis [161]. FDA-approved treatments include the HER2-specific antibodies trastuzumab and pertuzumab, which are used for passive immunotherapy and require repetitive administration [162, 163]. Passive immunization requires prolonged therapeutic delivery, cannot be developed as a prophylactic, and does not induce cellular immune responses [77, 78]. To overcome these challenges, HER-2$^+$ breast cancer research is now focused on active immunotherapy that elicits long-lasting cellular and humoral responses.

VLPs that display full-length HER-2 or specific immunogenic epitopes have been tested in clinical trials [77–81]. Peptides derived from the extracellular domain of HER-2 were incorporated into reconstituted influenzavirus virosomes (IRIVs) [164–166]. Three immunogenic peptides that are known to elicit B cell responses were tested: P4 (378–394), P6 (545–560), and P7 (610–623) [167]. The clinical trial revealed that 80% of vaccinated patients produced peptide-specific antibodies, and HER-2-specific IgG was elicited in 70% of the patients after immunization. A cellular immune response was also observed following

vaccination, involving the increased secretion of IL-2, TNFα, and IFNγ [77]. In a different approach, enveloped influenzavirus VLPs were modified to incorporate glycosylphosphatidylinositol (GPI)-anchored HER-2 (GPI-HER-2-VLP). Preclinical studies showed strong anti-D2F2/E2 (HER-2$^+$) serum IgG responses in mice, with comparable levels of IgG1, IgG2a, and IgG2b in the serum of vaccinated animals, indicating a balanced Th1 and Th2 response. In contrast, the vaccination of mice with soluble GPI-HER-2 predominantly elicited a Th2 response, whereas Th1-biased responses are needed to induce a potent anti-tumor reaction. When mice were challenged with HER-2$^+$ cells, those vaccinated with GPI-HER-2 VLPs showed a slower tumor growth rate compared to those administered GPI-HER-2 alone. 67% of the mice vaccinated with GPI-HER-2 VLPs remained tumor free [78].

VLPs have also been genetically modified to express HER-2 peptides. The internal face of the murine polyomavirus (MPyV) major capsid protein (VP1) can bind to the minor capsid protein (VP2) [168]. VP2 was genetically modified to express the N-terminal domain of HER-2, which contains the extracellular and transmembrane domains (VP2Her2$_{1–683}$). VP1 and VP2Her2$_{1–683}$ were produced in a baculovirus vector to obtain Her2$_{1–683}$PyVLPs. Immunized mice were challenged with HER-2$^+$ D2F2/E2 cells, and 87% of the vaccinated mice did not develop tumors. Similar results were obtained using transgenic BALB-neuT mice, which overexpress the rat HER-2 oncogene. The mice did not produce HER-2-specific antibodies, but did induce HER-2-specific T cells [79].

A T7 bacteriophage was genetically modified to express a H-2k^d-restricted cytotoxic T lymphocyte (CTL) epitope (p66) derived from rat HER-2 to investigate whether a CTL response is required for cancer immunotherapy. Preclinical studies showed that splenocytes from mice immunized with T7-p66 produced a higher IFNγ response than controls. Interestingly, splenocytes from mice vaccinated with a mixture of unconjugated T7 and p66 did not yield a strong IFNγ response when challenged with p66, indicating that the CTL peptide must be attached to T7 in order to elicit the CTL response. Splenocytes from mice vaccinated with T7-p66 were also able to lyse target cells pulsed with p66-peptide *in vitro*. Healthy mice vaccinated with T7-p66 rejected HER-2$^+$ TUBO cells, with five of the six mice remaining tumor-free 42 days after challenge. Furthermore, therapeutic vaccination with T7-p66 slowed the growth of pre-implanted tumors, eventually resulting in the full regression of HER-2$^+$ tumors [80].

We have recently worked on the development of a HER-2$^+$ breast cancer vaccine using the plant virus PVX. The chemical conjugation of PVX with the P4 B-cell epitope, which contains amino acids 387–394 from the extracellular domain of HER-2, elicited higher titers of HER-2-specific antibodies in mice than soluble P4 alone. These antibodies selectively recognized HER-2$^+$ breast cancer cells [81].

Addiction (cocaine and nicotine)

Addictive substances, such as nicotine and cocaine (Figure 11), affect the body by interacting with the nervous system. Nicotine and cocaine both modulate dopamine levels in the brain, thereby affecting the reward pathway. Vaccines against these addictive substances can help to reduce the severity of withdrawal symptoms and prevent relapse. However, small

molecules tend to have low immunogenicity, so VLP platform technologies are required to elicit potent and long-lasting immune responses against the drugs [169–171]. VLPs displaying nicotine or cocaine as multivalent arrays have been shown to elicit potent humoral responses, yielding drug-specific antibodies that prevent the substances from crossing the blood-brain barrier to exert their effects [172–174]. Nicotine is the addictive component of tobacco, and tobacco use is the leading preventable cause of disease, disability, and death in the industrialized world [172]. Several vaccines against nicotine are currently undergoing clinical trials, including NicVax™, NIC002, SEL-068, Ta-NIC, and IP18-KLH, but similar approaches are also under development for cocaine addition.

NIC002 is a VLP vaccine in which bacteriophage Qβ is chemically modified to display nicotine (NicQb) [82, 172, 174]. Preclinical studied in mice demonstrated development of nicotine-specific antibodies [172]. Importantly, when challenged with nicotine, vaccinated mice showed a higher concentration of nicotine remaining in the blood and a corresponding reduction of nicotine levels in the brain, compared to non-vaccinated mice [172]. In phase I trials, NicQb was immunogenic, well tolerated and efficacious in patients with high antibody titers, but the subsequent phase II trials did not support the primary endpoint of the study and the product was discontinued [82]. In an alternative approach, a nicotine analog was chemically linked to disrupted serotype-5 adenovirus (dAd5). The dAd5 VLP lacks the E1 and E3 proteins, allowing the particle to circumvent any pre-existing Ad5 immunity, which is prevalent in the population [83]. Sera from vaccinated mice contained high titers of anti-nicotine antibodies for a prolonged duration, resulting in lower concentrations of nicotine in the brain than naïve mice, inversely related to the levels of nicotine in the serum [83].

The dAd5 VLP has also been conjugated with the cocaine analog. Vaccinated mice challenged with cocaine were found to have 41% less cocaine in the brain than naïve mice. Locomotor activity in vaccinated mice challenged with cocaine was the same as in non-challenged mice treated with PBS, confirming that the vaccine reduced the cognitive impact of cocaine [84]. Similar strategies are being developed using alternative cocaine haptens [85].

Chronic diseases

Chronic diseases are persistent or even life-long conditions that require regular therapeutic intervention as a form of disease management. The prevalence of chronic diseases is increasing globally due to the ageing population and various dietary and lifestyle factors, which means that such diseases represent a significant and increasing public health burden in almost every country. Where chronic diseases are caused by malfunctioning self-proteins, vaccination can be used to induce the generation of autoantibodies. This approach has been tested in numerous disease models, including rheumatoid arthritis [175176–180], osteoporosis [177, 181], experimental autoimmune encephalitis [179], myocarditis [182], and obesity [183]. Many of these diseases are currently treated by passive immunotherapy, i.e. the regular administration of therapeutic antibodies targeting pathogenic self-proteins, which is expensive and restricts the patient. We will discuss hypertension and Alzheimer's disease as case studies for the alternative active immunization approach. The reader is recommended to consult further review articles for information about the development of vaccines against other chronic diseases[184, 185, 186].

Hypertension—Hypertension (high blood pressure) is an underlying risk factor that promotes the development of cardiovascular disease, which can lead to life threatening events such as heart attack and stroke. Although hypertension can be regulated with drugs, many hypertensive individuals never receive a diagnosis and therapeutic compliance tends to be poor even in diagnosed hypertensive patients. Another risk factor is the so-called morning pressure surge, a steep increase in blood pressure prior to waking, before medication can be taken. Active immunotherapy could overcome many of these challenges by inducing long-lasting immune responses targeting key regulators of blood pressure.

Angiotensin I and II could be the first targets of hypertension immunotherapy. These are small, soluble regulatory peptides (10 and 8 amino acids in length, respectively) which do not elicit a strong immune response in their native state. As described above, the immunogenicity of small molecules can be increased by exploiting VLP technology. A vaccine candidate has therefore been developed in which angiotensin II is chemically coupled to bacteriophage Qβ VLPs (AngQb). Preclinical studies in a rat model of hypertension showed that vaccination produced high titers of angiotensin II-specific IgGs and resulted in the normalization of blood pressure [187]. In clinical trials, the AngQb vaccine was well tolerated and no serious adverse effects were observed. The immunization of patients with mild to moderate hypertension reduced blood pressure during the daytime and especially in the early morning [188].

Alzheimer's disease—Alzheimer's disease is a neurodegenerative disorder characterized by a decline in cognitive ability accompanied by neuropathological features such as the loss of neurons in the hippocampus and neocortex and the accumulation of intracellular and extracellular protein deposits [189]. Extracellular protein deposits (amyloid plaques) contain the amyloid-β (Aβ) peptide, which is 42 amino acids in length [190, 191]. Previous reports have shown that immunization with the Aβ peptide reduced the deposition of amyloid plaques in transgenic mouse models [192]. Furthermore, passive immunization with Aβ antibodies had a similar effect [193]. However, a clinical trial (AN1792) using synthetic Aβ peptides for immunization showed minimal efficacy and was stopped after meningoencephalitis was reported in 6% of the subjects [194]. This unanticipated effect was attributed to a T-cell-mediated autoimmune response caused by the adjuvant QS21 [194–196]. The safety of Aβ-derived immunotherapies could therefore be improved by triggering a predominantly Th2-based immune response by delivering only B-cell epitopes, which are found on the N-terminus of the Aβ peptide [197, 198]. VLPs based on HPV, bacteriophage Qβ, HBc, and BPV-1 have already been used to display Aβ peptides [86, 89, 90].

HPV-16 displaying Aβ peptides such as full-length Aβ ($Aβ_{1–40}$), N-terminal Aβ ($Aβ_{1–9}$ and $Aβ_{1–16}$), mid-domain Aβ ($Aβ_{12–28}$), and C-terminal Aβ ($Aβ_{17–40}$) have been tested as vaccines. HPV-$Aβ_{1–40}$ elicited IgG responses without the use of Freund's adjuvant in mice, whereas free $Aβ_{1–40}$ required Freund's adjuvant to obtain comparable titers. HPV conjugated to peptides from the N-terminal domain of Aβ elicited higher antibody titers than HPV conjugated to peptides from either the mid or C-terminal domains, indicating that N-terminal Aβ peptides are the most immunogenic when presented on HPV particles. Importantly, these antibodies were predominantly of subtype IgG1, indicating a Th2-biased immune response [86].

Aβ peptides have been directly conjugated to bacteriophage Qβ. The N-terminal modified peptide ($Aβ_{1–9}$) with a C-terminal –GGC linker was conjugated to the bacteriophage using a bifunctional linker with both amine and sulfhydryl reactive arms (SMPH). Mice immunized with Qβ-$Aβ_{1–9}$ VLPs without adjuvant produced higher titers of antibodies than those immunized with HPV-$Aβ_{1–9}$ and similar titers to those immunized with HPV-$Aβ_{40}$. The inclusion of incomplete Freund's adjuvant increased the IgG titers even further [86].

Bacteriophage Qβ has also been conjugated with $Aβ_{1–6}$, which is shorter than the typical T-cell epitope. Mice were immunized three times with Qβ-$Aβ_{1–6}$ and did not activate Aβ-specific T cells. Additionally, mice immunized with Qβ-$Aβ_{1–6}$ produced high antibody titers against the Aβ peptide and formed fewer plaques than control mice [199]. Qβ-$Aβ_{1–6}$ was tested in a phase I trial (CAD106) and was deemed safe and tolerable in a double-blind, placebo-controlled, 52-week study. Importantly, no subjects recorded clinical or subclinical cases of meningoencephalitis, which halted the previous Alzheimer's disease immunotherapy clinical trial [88]. In a phase II trial, CAD106 was administered to 47 patients with Alzheimer's disease (n = 11 for placebo). The patients received three subcutaneous or intramuscular doses of CAD106, followed by four additional subcutaneous or intramuscular injections. Long-term treatment induced prolonged high titers of Aβ-specific antibodies suggesting that CAD106 could be developed into an effective immunotherapy for Alzheimer's disease [87].

A C-terminally truncated version of the HBc protein (HBcΔ) was genetically modified to include two copies of $Aβ_{1–15}$ in the MIR (Aβ-HBc) [89]. The MIR was chosen because epitopes inserted there tend to be highly antigenic and immunogenic compared to other insertion sites [200]. Preclinical studies in mice showed that anti-Aβ antibodies, predominantly IgG1 and IgG2b subtypes (indicating a Th2-biased immune response) were developed. Free Aβ peptide with adjuvant also elicited high antibody titers, but was dominated by the IgG2a subtype. Sera from immunized mice also prevented formation of Aβ fibrils and reduced the toxicity of the Aβ peptide towards PC12 cells [89].

In the final example based on BPV-1, the $Aβ_{1–9}$ peptide was fused with the L1 protein and the chimeric Aβ-VLPs self-assembled to form a structure resembling the native virus particle. Preclinical vaccination studies in rabbits indicated that sera from the treated rabbits recognized $Aβ_{1–9}$ and full length Aβ, and that Aβ fibril formation was inhibited *in vitro*. Transgenic APP/PS1 mice, which spontaneously form Aβ plaques, were also immunized with Aβ-VLP without adjuvant eliciting high titers of Aβ-specific antibodies. Higher levels of circulating Aβ peptide were detected in these mice compared to naïve controls, corresponding to lower levels of Aβ peptide in the brain [90].

Conclusion

Vaccines based on viruses could be developed for the prevention and/or treatment of diverse diseases, including infectious diseases, cancer, addiction, and chronic disorders. One of the key advantages of viruses as a vaccine development platform is that they are naturally immunogenic, and are therefore ideal for the induction of immune responses even in the absence of an adjuvant. The success of prophylactic vaccines against HPV and HBV

highlights the potential of this platform for the treatment of many other diseases. Many virus-based vaccines have shown promising results in non-human primates but a number of challenges remain to be overcome before such vaccines can be deployed in the clinic. The first barrier is safety: the natural immunogenicity of virus-based particles makes them ideal for the display of antigenic epitopes but increases the risk of toxicity. Bacteriophage Qβ vaccines for Alzheimer's disease and nicotine addiction have recently completed phase I safety tests, but other platforms remain to be evaluated in clinical trials. Nevertheless, the phase I trials indicate that virus-based vaccines offer an alternative to other vaccine materials, and the promising results in primates indicate that this platform could be used to develop novel vaccines against a wide range of diseases.

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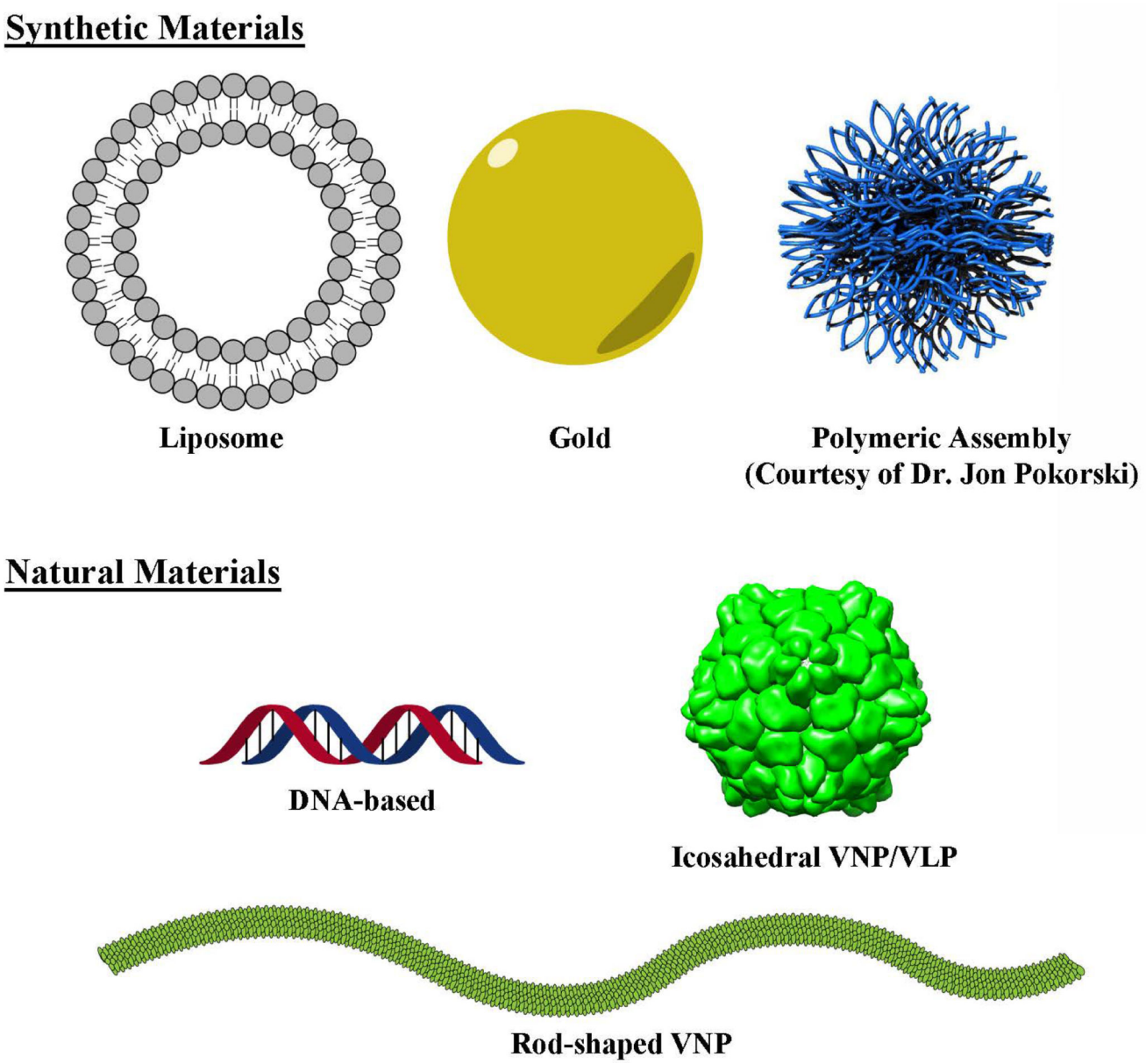

Figure 1.
Virus-based nanoparticles (VNPs) as platform technologies for vaccine development.

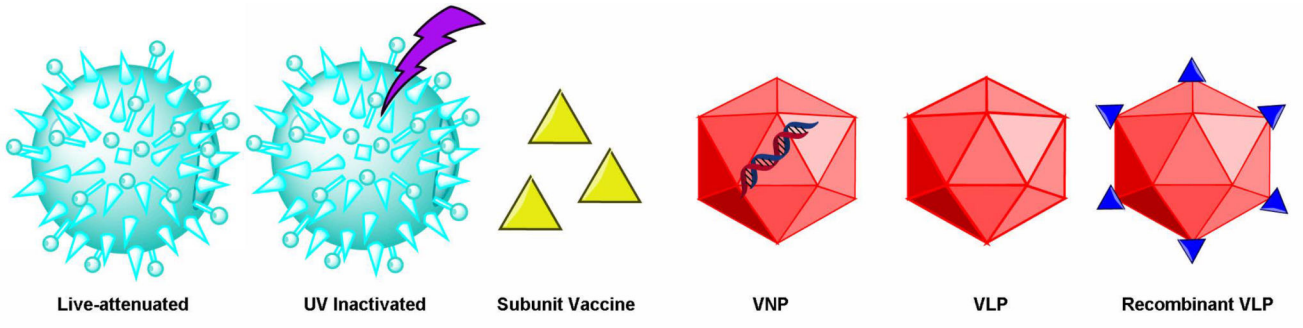

Figure 2.
Categories of viral vaccines; UV = ultraviolet; VNP = viral nanoparticle; VLP = virus-like particle

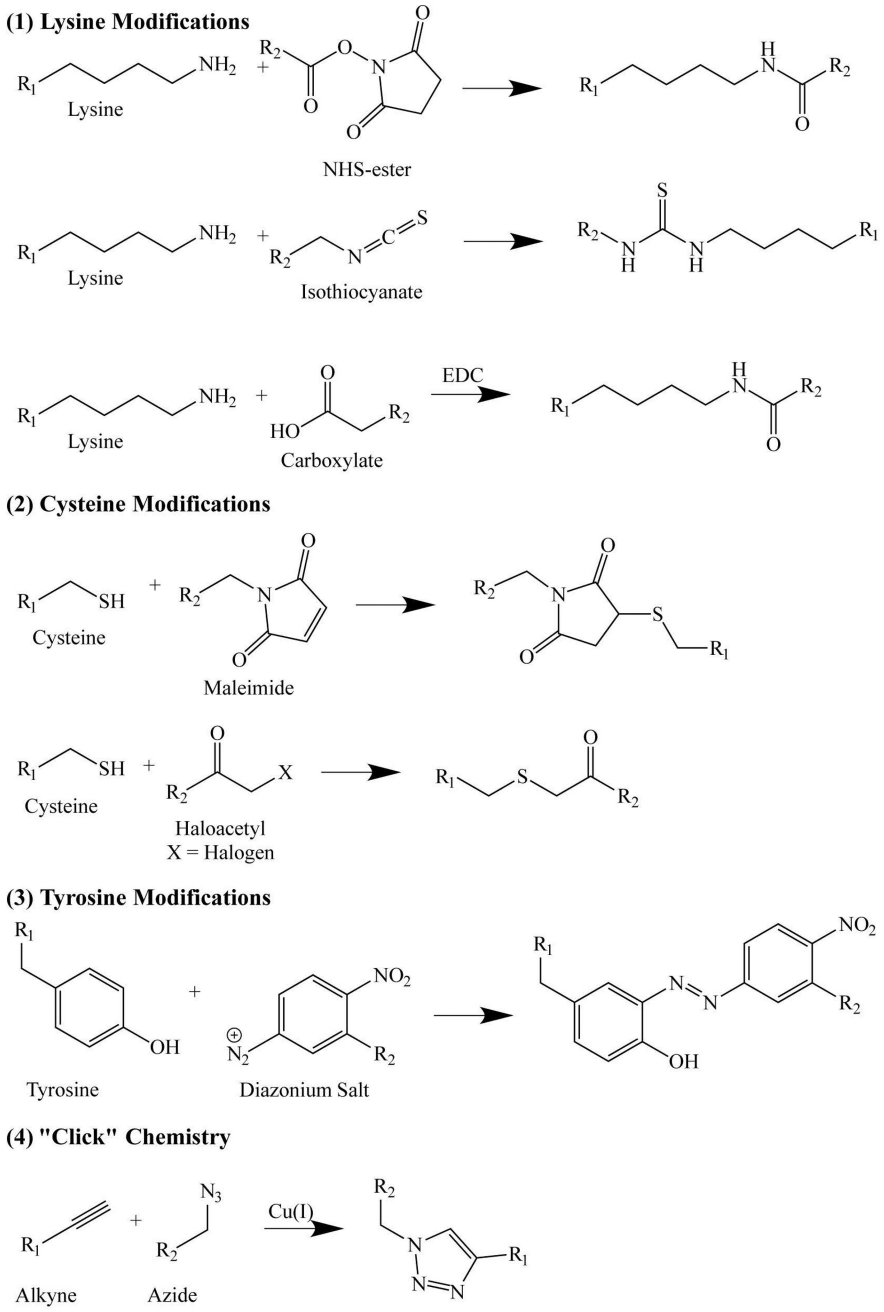

Figure 3.
Chemical conjugation strategies (bioconjugation); NHS = *N*-hydroxysuccinimde

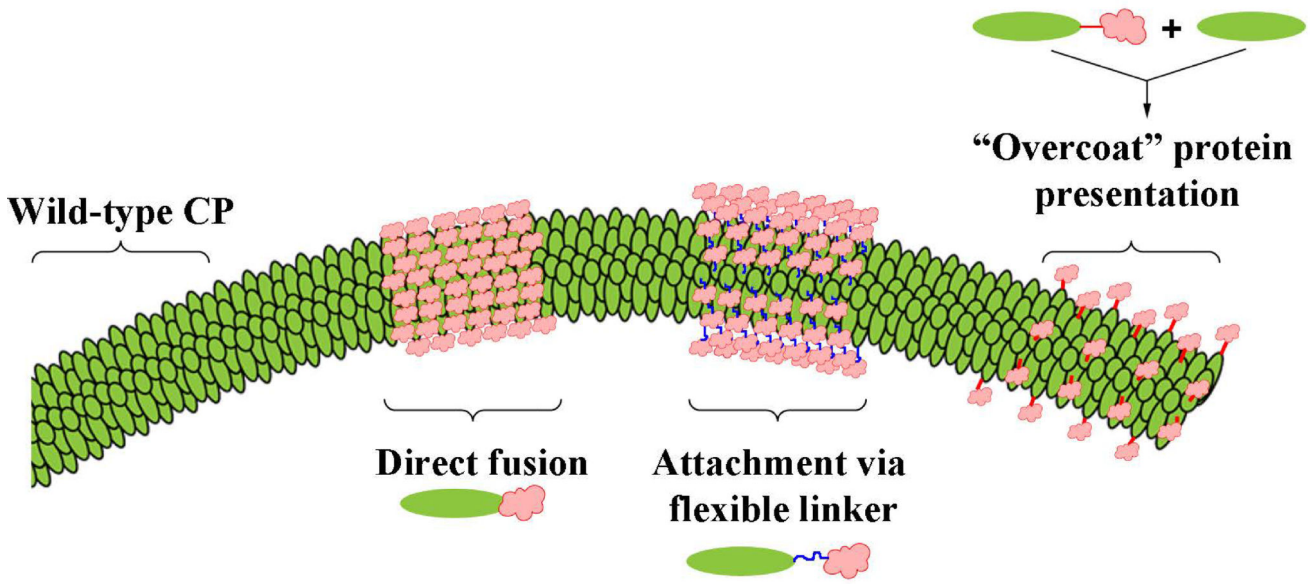

Figure 4.
Genetic engineering strategies for the display of epitopes on viral coat proteins.

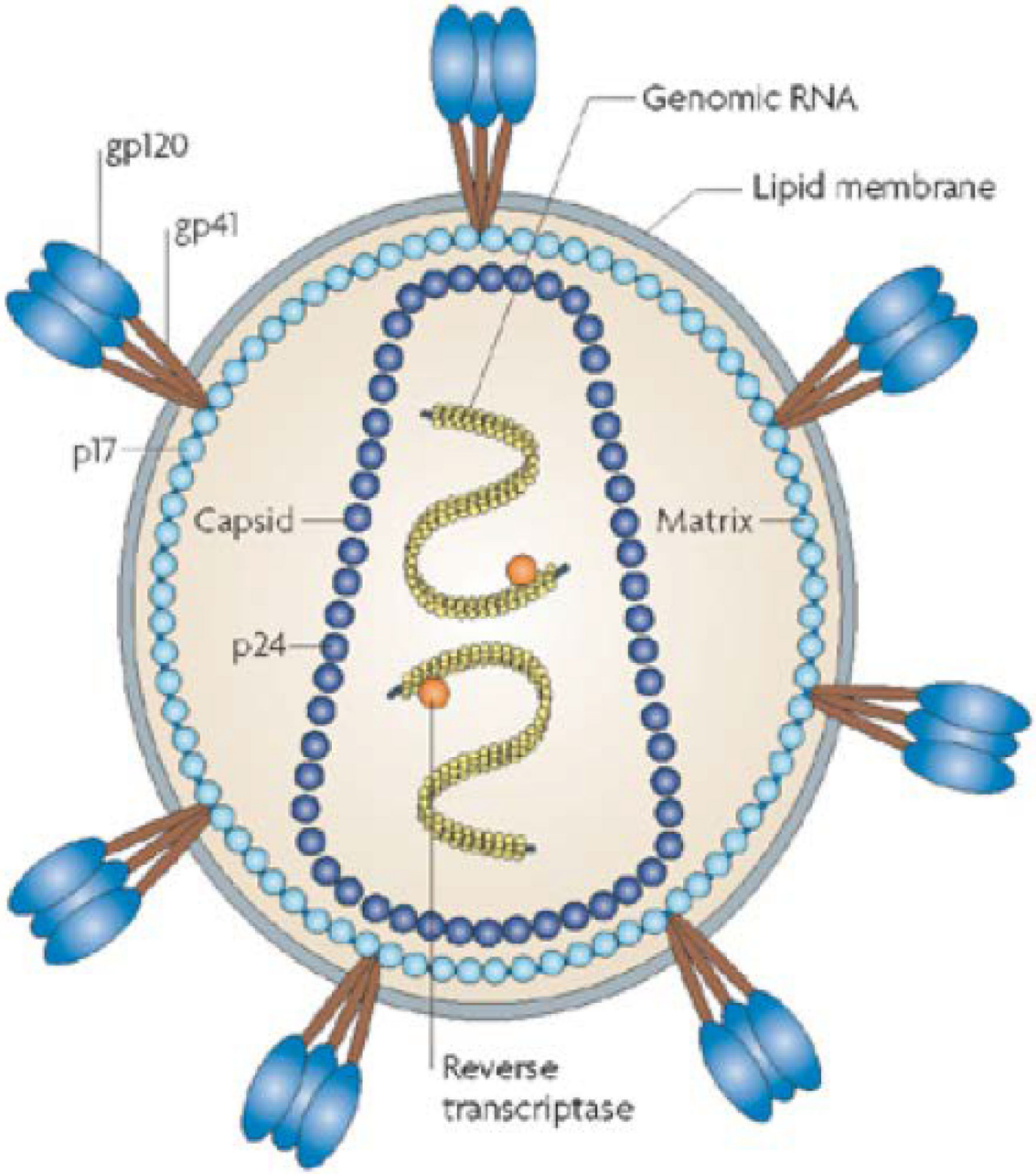

Figure 5.

In HIV-1, trimeric gp120-gp40 complexes are embedded in the membrane. The transmembrane glycoprotein gp41 and the external envelope glycoprotein gp120 are depicted in non-covalent association. The cytoplasmic tail of gp41 interacts with the HIV-1 matrix protein p17. The capsid protein, p24, makes up the cone-shaped core, which contains two positive-strand RNA copies of the HIV-1 genome that are surrounded by the nucleocapsid protein (yellow). Reverse transcriptase protein is also packaged into the particle. (Adapted by permission from Macmillan Publishers Ltd: *Nature Reviews Microbiology*; Ref 91 Copyright 2008)

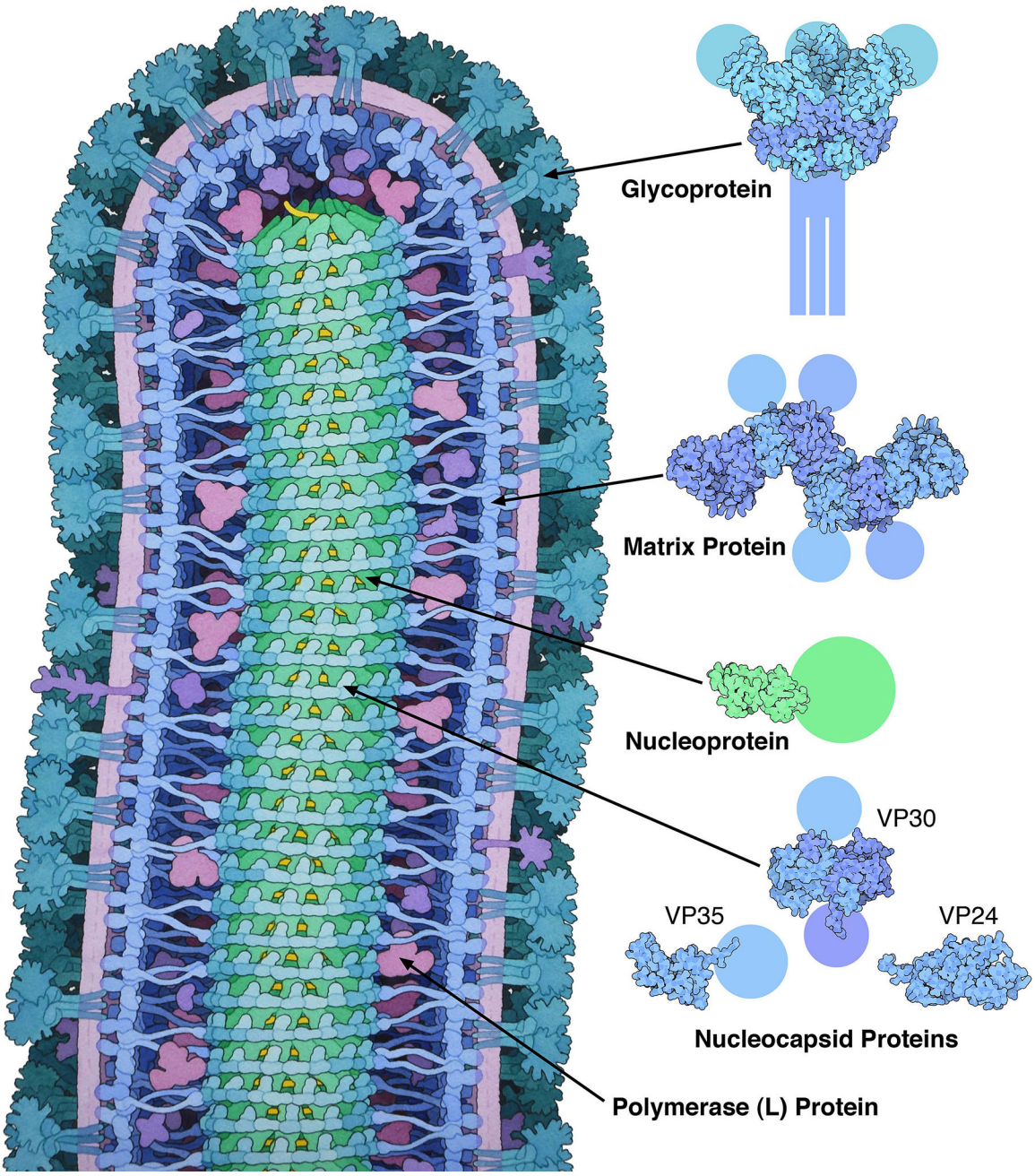

Figure 6.
Structure of *Ebola virus* (Courtesy of David S. Goodsell and RCSB PDB)

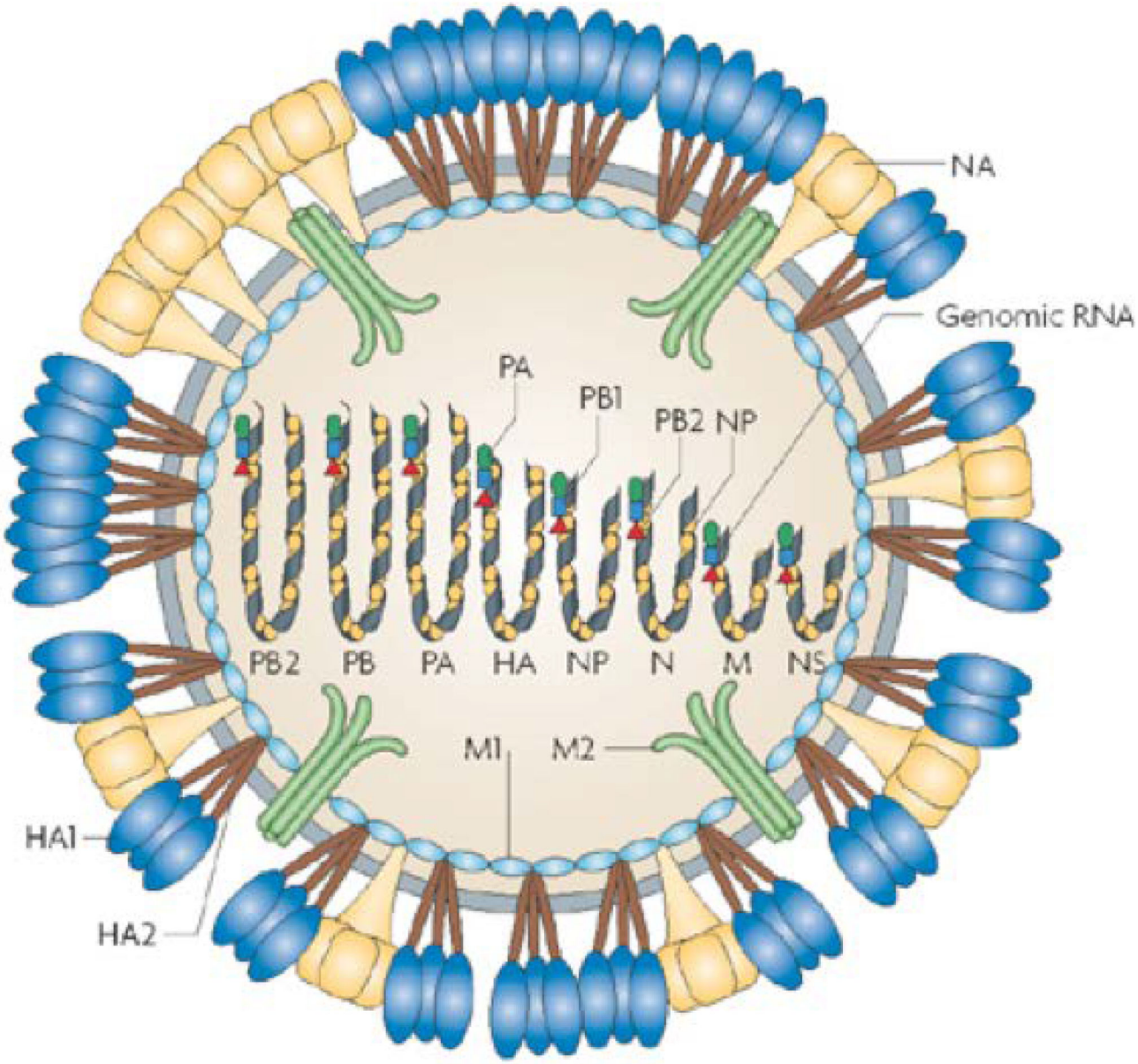

Figure 7.

In influenza A virus, 3 viral proteins are exposed on the outside of virus particles: haemagglutinin (HA, which forms trimers), neuraminidase (NA) (which forms tetramers) and M2 (which forms tetramers that make up ion-channels). Upon proteolytic cleavage, HA0 (not shown) is processed to HA1 and HA2. The influenza virus matrix protein M1 associates inside the viral membrane, and the viral genome consists of eight negative-strand RNA segments and is packaged into the particle as a ribonucleoprotein in complex with nucleocapsid protein (NP) and the viral polymerases PA, PB1 and PB2. (Adapted by permission from Macmillan Publishers Ltd: *Nature Reviews Microbiology*; Ref 91 Copyright 2008)

Figure 8.
Production facility at Medicago

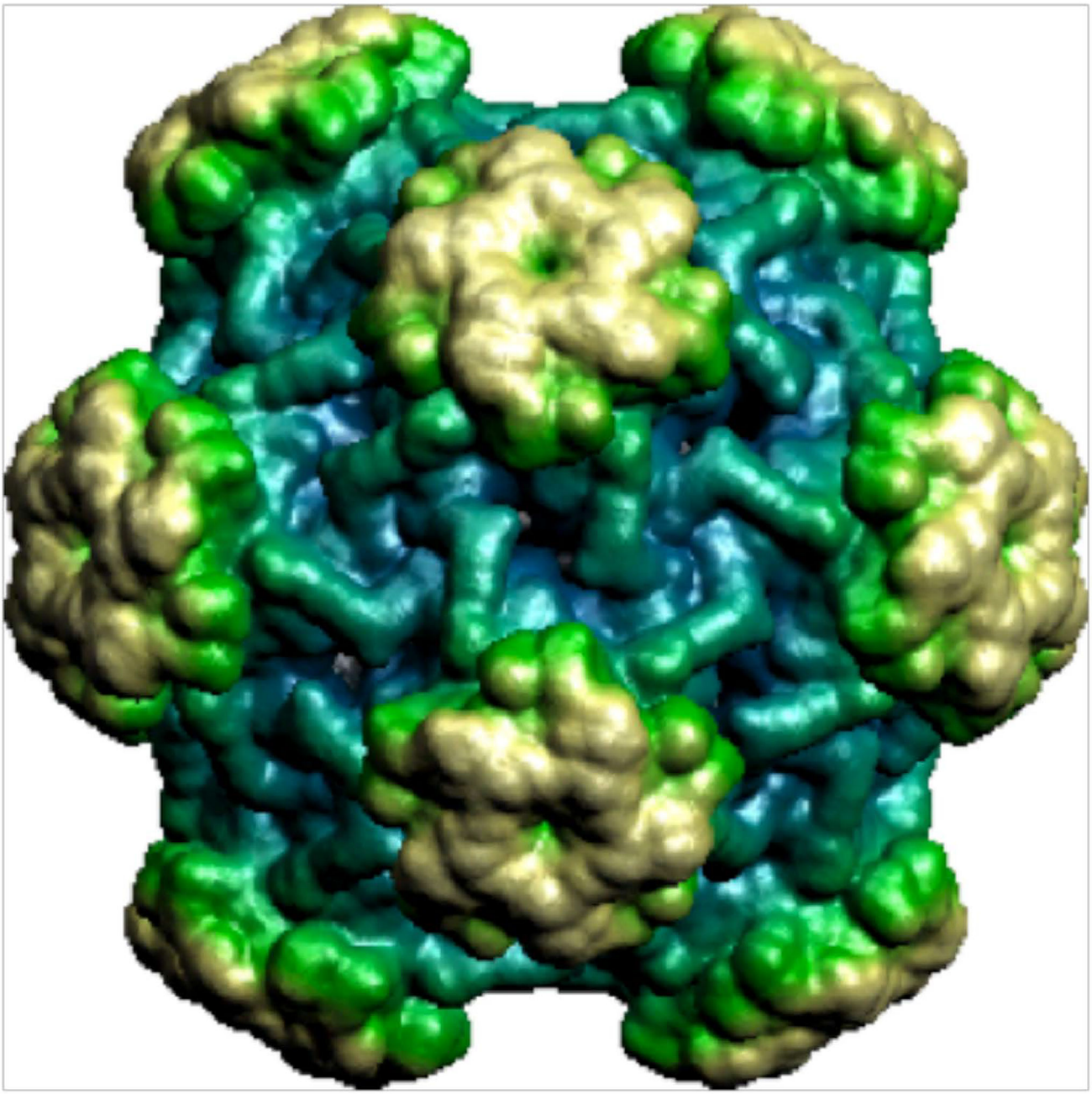

Figure 9.
Human papillomavirus 16 L1 capsid (viperdb.scripps.edu)

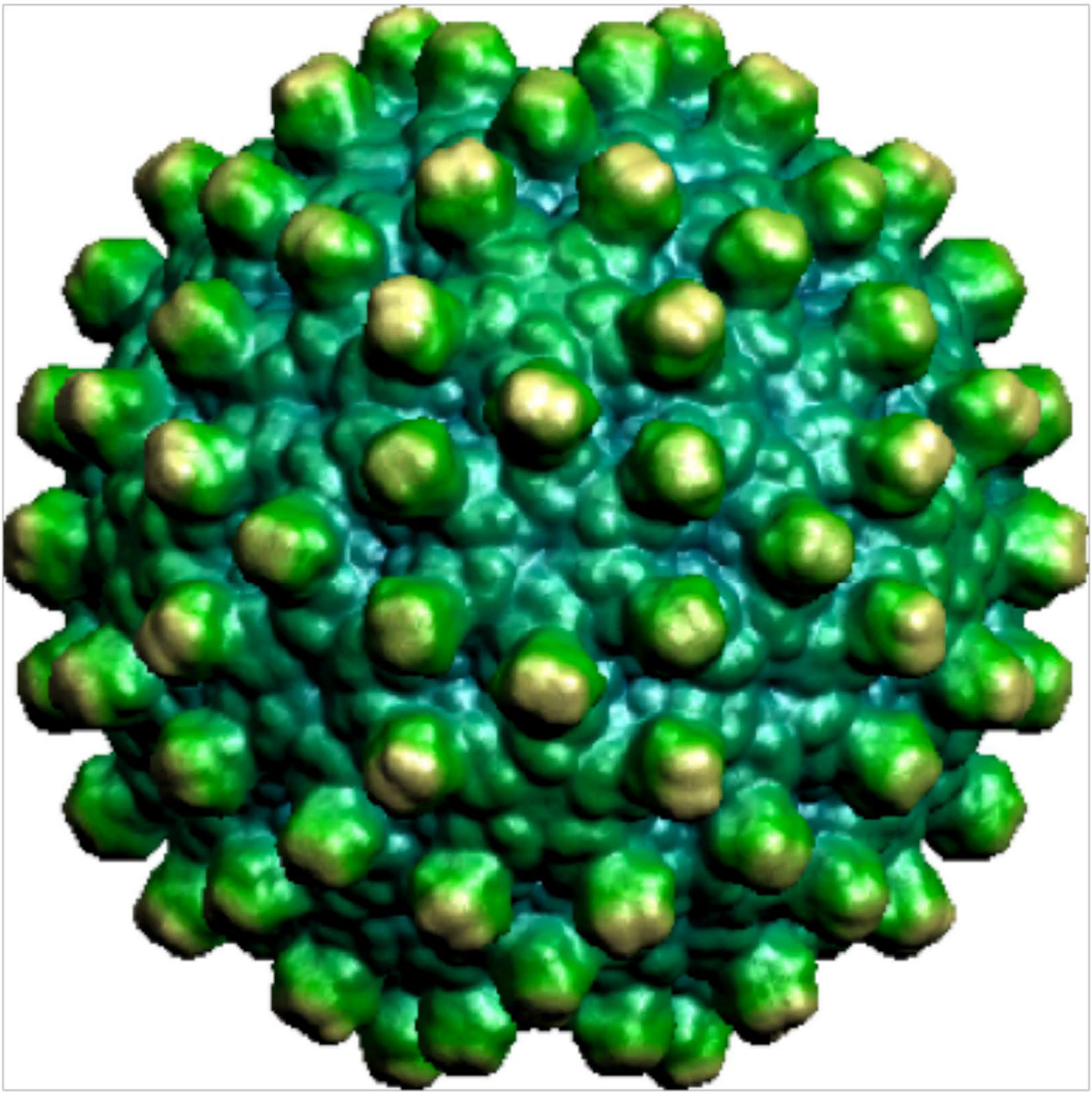

Figure 10.
Human *Hepatitis B virus* (viperdb.scripps.edu)

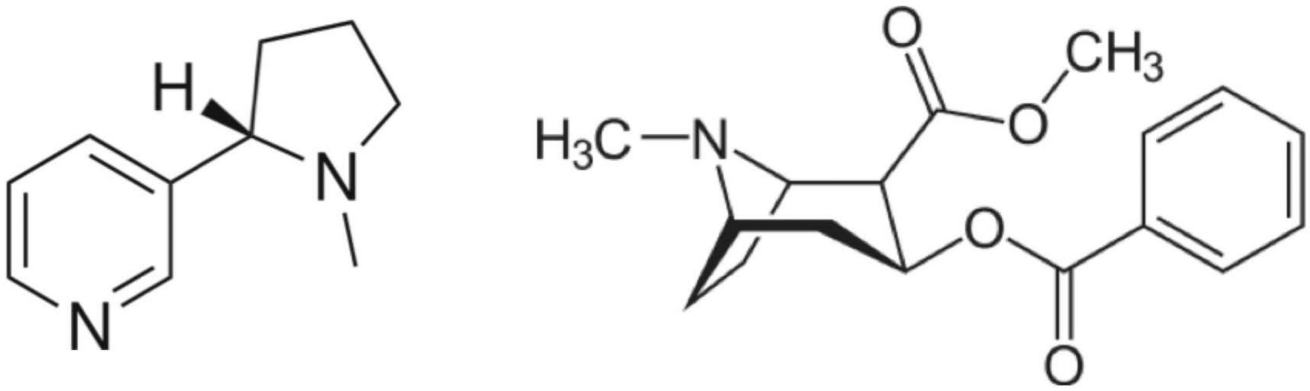

Figure 11.
Chemical structures of nicotine (left) and cocaine (right).

Table 1

Key VLP/VNP-based vaccines approved or under development.

Vaccine Target	Platform	Composition	Stage of Development	References
HIV	AP205	gp41 epitope	Animal studies	36
HIV	PVX	gp41 epitope	Animal studies	27
HIV	BPV-1	CCR5 peptide	Animal studies	37
HIV	Qβ	CCR5 peptides	NHP studies	38
HIV	Canarypox	Env, gag, pol genes + AIDSVAX (gp120)	Testing in humans	39–43
HIV/SIV	RABV	SIV envelope	Animal studies	44
HIV/SIV	Virosomes	gp41 epitopes	NHP studies	45
Ebola	Ebola	VP40 and GP	Animal studies	46, 47
Ebola	Ebola	GP, NP, and VP40	NHP studies	48
Ebola	Ebola	EBOVΔVP30	NHP studies	49
Ebola	rVSV	GP	Testing in humans	50
Ebola	RABV	GP	NHP Studies	51, 52
Sudan virus and Marburg Virus	RABV	GP	Animal studies	52
Influenza (pandemic)	Influenza	HA, NA, and M1 from H1N1	Animal studies	53
Influenza (pandemic)	Influenza	HA and NA from H7N9; M1 from H5N1	Animal studies	54
Influenza (pandemic)	Influenza	HA and NA from H1N1; M1 from H5N1	Testing in humans	55
Influenza (pandemic)	Influenza	H5 and H1	Testing in humans	56
Influenza (universal vaccine)	Influenza	HA, NA, and M1 from H5N1	Animal studies	57
Influenza (universal vaccine)	HBc	HA	Animal studies	58
Influenza (universal vaccine)	Dd	M1	Cell studies	59
Influenza (universal vaccine)	PapMV	M2e	Animal studies	60
Influenza (universal vaccine)	IBDV	HA and M2 from H1N1	Animal studies	61
Influenza (universal vaccine)	PapMV	NP	Animal studies	62
Influenza (universal vaccine)	PVX	NP	Animal studies	63
Influenza (universal vaccine)	P22	NP	Animal studies	34
Influenza (universal vaccine)	sHSP	n/a	Animal studies	64, 65
Leukemia	TMV	TACA	Animal studies	66
Leukemia	Qβ	TACA	Animal studies	67
Melanoma	TMV	p15e and Trp2	Animal studies	68
Lymphoma	PVX	Id	Animal studies	69
Ductal adenocarcinoma	BPV	MUC-1	Animal studies	70
HPV (prophylactic)	HPV-16, −18	L1	Clinically available	Cervarix
HPV (prophylactic)	HPV-6, −11, −16, −18	L1	Clinically available	Gardasil®
HPV (prophylactic)	MS2	L2 from HPV-16 and −31	Animal studies	71, 72
HPV (prophylactic)	PP7	L2 from HPV-1, −16, and −18	Animal studies	72
HPV (prophylactic)	HPV-18 L1 VLP	L2 from HPV-18, −45, and −59	Animal studies	73

Vaccine Target	Platform	Composition	Stage of Development	References
HPV (therapeutic)	HPV-16	E7	Testing in humans	[74]
HPV (therapeutic)	HPV-16 L1 VLP	E6 and E7	Animal studies	[75]
HBV (prophylactic)	HBV	HBsAg	Clinically available	Recombivax HB
HBV (therapeutic)	HBc	HBx-derived cytotoxic T lymphocyte epitopes and PADRE	Animal studies	[76]
HER-2$^+$ breast cancer	IRIV	P4, P6, and P7	Testing in humans	[77]
HER-2$^+$ breast cancer	Influenza	GPI-HER-2	Animal studies	[78]
HER-2$^+$ breast cancer	MPyV	HER-2$_{1–683}$	Animal studies	[79]
HER-2$^+$ breast cancer	T7	p66	Animal studies	[80]
HER-2$^+$ breast cancer	PVX	P4	Animal studies	[81]
Nicotine addiction	Qβ	Nicotine	Testing in humans	[82]
Nicotine addiction	dAd5	AM1	Animal studies	[83]
Cocaine addiction	dAd5	GNC	Animal studies	[84]
Cocaine addiction	dAd5	GNE	Animal studies	[85]
Hypertension	Qβ	Angiotensin II peptide (8 aa)	Testing in humans	[28]
Alzheimer’s disease	HPV-16	Aβ peptides	Animal studies	[86]
Alzheimer’s disease	Qβ	Aβ$_{1–9}$	Animal studies	[86]
Alzheimer’s disease	Qβ	Aβ$_{1–6}$	Testing in humans	[87, 88]
Alzheimer’s disease	HBcΔ	Aβ$_{1–15}$	Animal studies	[89]
Alzheimer’s disease	BPV1	Aβ$_{1–9}$	Animal studies	[90]