Part IV
Applied Structural and Physical Virology

Chapter 20
Antiviral Agents: Structural Basis of Action and Rational Design

Luis Menéndez-Arias and Federico Gago

Abstract During the last 30 years, significant progress has been made in the development of novel antiviral drugs, mainly crystallizing in the establishment of potent antiretroviral therapies and the approval of drugs inhibiting hepatitis C virus replication. Although major targets of antiviral intervention involve intracellular processes required for the synthesis of viral proteins and nucleic acids, a number of inhibitors blocking virus assembly, budding, maturation, entry or uncoating act on virions or viral capsids. In this review, we focus on the drug discovery process while presenting the currently used methodologies to identify novel antiviral drugs by using a computer-based approach. We provide examples illustrating structure-based antiviral drug development, specifically neuraminidase inhibitors against influenza virus (*e.g.* oseltamivir and zanamivir) and human immunodeficiency virus type 1 protease inhibitors (*i.e.* the development of darunavir from early peptidomimetic compounds such as saquinavir). A number of drugs in preclinical development acting against picornaviruses, hepatitis B virus and human immunodeficiency virus and their mechanism of action are presented to show how viral capsids can be exploited as targets of antiviral therapy.

Keywords Antiretroviral drugs • Capsid proteins • DNA polymerases • Drug development • Fusion inhibitors • Hepatitis virus • Herpesviruses • Human

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immunodeficiency virus • Influenza virus • Ligand docking • Neuraminidase • Nucleoside analogues • Proteases • Viral assembly • Viral entry • Viral replication • Virtual screening

Abbreviations

AIDS	Acquired immune deficiency syndrome
CoMFA	Comparative molecular field analysis
CTD	C-terminal domain
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HR	Heptad repeat
HSV	Herpes simplex virus
HTS	High-throughput screening
LBVS	Ligand-based virtual screening
mRNA	Messenger RNA
Neu5Ac	*N*-acetylneuraminic acid
NMR	Nuclear magnetic resonance
NNRTIs	Nonnucleoside RT inhibitors
NTD	N-terminal domain
PDB	Protein Data Bank
RSV	Respiratory syncytial virus
RT	Reverse transcriptase
SBVS	Structure-based virtual screening
THF	Tetrahydrofuran
VZV	Varicella-zoster virus

20.1 Introduction

Antiviral drugs are compounds that stop the development and propagation of a virus without causing a relevant damage in the host cell. Despite landmark achievements (*e.g.* >30 new drugs approved during the last three decades to fight AIDS), the number of available antiviral compounds is still small and effective only against a limited group of pathogens. Examples are human immunodeficiency virus (HIV), herpes simplex virus (HSV), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), influenza virus and hepatitis B and C viruses (HBV and HCV, respectively) [1].

There are several reasons that account for the difficulties in developing antiviral agents. First, viruses are obligatory intracellular parasites. Because every step in the

viral life cycle engages host functions, it is difficult to interfere with virus growth without having a negative impact on the host cell. Side effects are relatively common. Second, clinically important viruses are often dangerous and cannot be propagated or tested in model systems. Thus, viruses causing fatal diseases in humans (*e.g.* smallpox or several hemorrhagic diseases) have to be handled by well-trained and experienced scientists, and in facilities with strict containment requirements. Not surprisingly, these labs are expensive and difficult to maintain. Apart from the biological safety limitations, sometimes viral infections cannot be properly monitored for antiviral drug development due to the lack of appropriate animal models of human disease (*e.g.* smallpox or measles) or to difficulties in growing the virus in cell culture (*e.g.* HBV).

A third factor that limits the efficacy of antiviral drugs is their potency requirements. Ideally, an antiviral agent should be extremely potent. Partial inhibition is not acceptable for an antiviral drug. The reason is that limited viral replication under drug pressure allows for the generation of variants that can be selected under treatment. The emergence of resistance is a major drawback of many antiviral therapies. For example, in the case of HIV, therapies prescribed in the late 1980s or early 1990s were based on a single drug (mostly zidovudine) or combinations of two drugs (usually two inhibitors of the viral polymerase) [2]. However, those drugs were not potent enough to limit the emergence of drug-resistant variants [3] and therefore these viruses were almost impossible to combat successfully with the available drug armamentarium. In a patient with full blown AIDS, HIV production has been estimated at about 10^{12} virions per day. The high replication rate of HIV and the low fidelity of its DNA polymerase [4] trigger the appearance of drug resistance under suboptimal therapies.

Another issue that deserves some attention is the short duration of many viral infections (*e.g.* flu, common cold, etc. . . .). Very often, the symptoms appear when the virus is no longer replicating and are due to the immune response of the patient. In those cases, antiviral drugs should be administered early in infection or as a prophylactic measure in populations at risk. However, this could be potentially harmful for healthy populations.

20.2 Drug Discovery and Potential Targets of Antiviral Intervention

Early evidence of activity against vaccinia virus was reported for several thiosemicarbazone derivatives [5], and one of them (N-methylisatin-β-thiosemicarbazone) entered clinical studies for the prophylaxis of smallpox [6] at the time when vaccination against smallpox virus took over. The first antiviral drug licensed for clinical use was a thymidine analogue known as idoxuridine (5′-iodo-2′-deoxyuridine), whose synthesis was described in 1959 [7]. Idoxuridine has been used topically to treat eye and skin infections caused by herpes simplex virus. The drug acts on viral

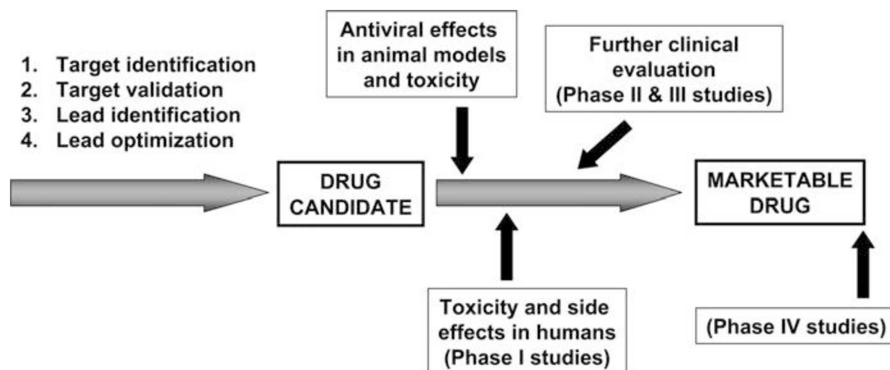

Fig. 20.1 Schematic outline of the drug discovery process

replication by interfering with the normal function of thymidylate phosphorylase and viral DNA polymerases.

Encouraged by the success with antibiotics in the 60s and 70s, drug companies launched huge blind-screening programs that were not very fruitful. Today, recombinant DNA technology and sophisticated chemistry [8], as well as impressive advances in structural and functional genomics, have facilitated the identification and analysis of particular proteins or mechanisms. Essential viral genes can be cloned and expressed in appropriate organisms so that the encoded proteins can be purified and analyzed in molecular and atomic detail.

Drug discovery programs start with the identification of suitable drug targets (Fig. 20.1). A drug target can be defined as a biomolecule (usually a single protein or a protein complex) linked to a disease and containing a suitable binding site that can be exploited to modulate its function. These targets need to be validated to demonstrate that they are critically involved in a disease and that their modulation is likely to have a therapeutic effect. In virology, many drug targets are viral proteins (*e.g*. enzymes), nucleic acids or other biological macromolecules required in the virus life cycle. Infection and viral propagation can be blocked by small compounds binding to relevant targets. Once the target has been validated *in vitro* and/or in animal models, lead identification starts with the design and development of a suitable assay to monitor the biological function under study. Active compounds that demonstrate dose-dependent target modulation and some degree of selectivity for the target under study are called lead compounds. These molecules are optimized in terms of potency and selectivity to become drug candidates.

In order to become a marketable drug, the candidate undergoes additional preclinical evaluation, including pharmacokinetic and toxicity studies in animal models. Clinical trials involving new drugs are commonly classified into four phases. Approval is usually granted if the drug advances successfully through phases I, II and III. The main objective of phase I is to assess drug safety and pharmacokinetics in a relatively small number of healthy individuals who receive small doses of the compound. In phase II, the testing protocol is established. These are trials trying to find out

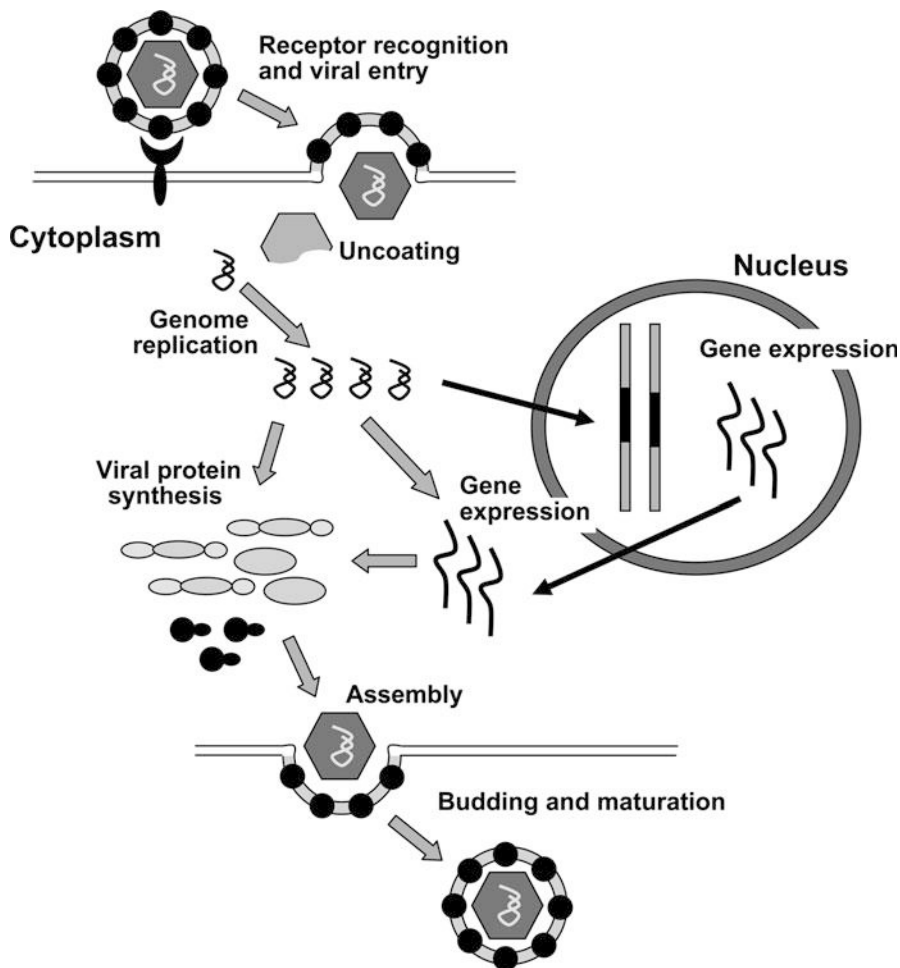

Fig. 20.2 Basic steps of viral replication as potential targets of antiviral therapy

appropriate doses, prescription regimens, etc.... and are carried out with patients. Phase III constitutes the final testing before approval and these trials try to show whether the drug has a measurable benefit or advantage over other treatments with a relatively large number of patients (usually >100 patients for antiviral drugs). Phase IV includes all studies carried out after approval of the drug and may address many different issues, from side-effects to efficacy in comparison with other drugs or regimens, and usually involve a large number of patients worldwide.

Recent years have witnessed a remarkable progress in the discovery and development of antiviral agents, fueled by advances in the understanding of viral life cycles which in turn have provided new opportunities for therapeutic intervention. Although the mechanisms of replication and propagation can show significant variations between different viruses, a prototypic life cycle is presented in

Fig. 20.2 (see also Chap. 1). Typically, a virion (*i.e.* an infectious viral particle) first attaches to the surface of the host cell. This interaction can be specific and involve the participation of one or more different types of proteins. For example, in HIV-1, the main receptor is CD4, but other proteins (*e.g.* chemokine receptors CCR5, CXCR4, etc. . . .) can facilitate viral entry acting as coreceptors (see Chap. 15).

Viral uptake occurs through different mechanisms such as endocytosis (as for example, in the case of influenza virus) or the fusion of the cellular membrane with the viral envelope, as demonstrated in the case of retroviruses (see Chap. 16). These events allow the internalization of the capsid containing the viral genome. Uncoating or disassembly is a still poorly understood process that releases the viral genome from its protein shell (see Chaps. 15 and 16). Disassembly may occur rapidly after fusion, as occurs in HIV, or be triggered by pH variations, as observed in viruses entering the cell by an endocytic pathway (*e.g.* influenza virus). The replication of the viral genome occurs by different mechanisms depending on whether the viral genome is a single- or double-stranded DNA or RNA. In general, DNA viruses use cellular pathways to replicate their genomes, but RNA viruses (and in general, those replicating in the cytoplasm) provide their own enzymes to complete virus replication. Cellular factors, together with specific proteins encoded within the viral genome, contribute to transcription and post-transcriptional modification of viral messenger RNAs (mRNAs). In some viruses (for example, in retroviruses), viral mRNAs are generated in the nucleus by the action of the cellular RNA polymerase. This is due to the integration of the viral genome (as a double-stranded DNA) into the genome of the host cell.

Viral polyproteins are synthesized by the cellular translational machinery. After post-translational modification, viral proteins and their genomes are transported to assembly sites within the host cell (see Chap. 14). Viral factors may also participate in these processes. The sites of assembly are frequently located at the plasma membrane or in intracellular factories (often associated with membranes). Virion assembly is a complex process which involves multiple molecular recognition steps and conformational transitions. The viral capsid is assembled in a multimerization reaction, with or without the help of scaffolding proteins or viral nucleic acids (see Chaps. 10, 11 and 19). The viral nucleic acids are packaged into the capsid during or after its assembly (see Chap. 12). Finally, the viral particle can be transformed into an infectious virion through a maturation process that involves changes in the structure and properties of the capsid (see Chap. 13).

In enveloped viruses, a membrane with embedded viral proteins is incorporated into the virion (see Chap. 11), and the resulting viral particles are released after budding. In contrast, cell lysis mediates the release of non-enveloped viruses. In some cases (*e.g.* in retroviruses), maturation occurs once the immature virion has been released and involves the proteolytic processing of precursors containing the viral proteins, including those that form the capsid.

All of those steps of the viral life cycle constitute potential targets of antiviral intervention. At present, drugs inhibiting viral enzymes involved in the replication or expression of the viral genome are commonly used in antiviral treatments. However, recent developments including the determination of structures, properties and functions of capsids and virions, as well as the elucidation of events involving

interactions between components of the viral particle or between them and host cell molecules (the subjects of this book) have opened novel avenues for the design of drugs acting directly on the viral particle. This chapter provides examples of approved or pre-clinical antiviral strategies directed at inhibiting viral nucleic acid metabolism, as well as others aimed at interfering with cell recognition, entry, uncoating, assembly, or maturation of virus particles.

20.3 Antiviral Drugs and Mechanisms of Action

Licensed compounds used in the treatment of viral infections target HIV, HBV, HCV, influenza virus, HSV, and other herpesviruses such as VZV and HCMV. A number of drugs act on steps that lead to the formation of the viral capsid or the mature virion (*i.e*. assembly, budding and maturation) while others, whose target is the assembled capsid or the virion, interfere with processes affecting viral entry and uncoating [1]. Nonetheless, most of the approved drugs block intracellular events affecting the synthesis and dynamics of viral proteins and nucleic acids (Table 20.1). Within this group, viral polymerases constitute the major target for many antiviral drugs.

20.3.1 Drugs Blocking Intracellular Processes Required for the Synthesis of Viral Components

Viral Genome Replication Inhibitors

Compounds inhibiting the replication of HSV, VZV and HCMV include prodrugs of nucleoside analogues (*e.g*. valacyclovir, valganciclovir and famciclovir) that need to be phosphorylated in order to become substrates of the viral DNA polymerase (Fig. 20.3). Viral enzymes (*e.g*. thymidine kinases in HSV and VZV, and a protein kinase in HCMV) are responsible for the transformation of acyclovir, ganciclovir and penciclovir into their monophosphate derivatives. Further phosphorylation steps are carried out by host cell kinases. The triphosphate derivatives of acyclovir, ganciclovir and penciclovir that mimic the natural substrates of the viral DNA polymerase, are incorporated into the growing DNA chain and often terminate viral replication. because they lack a 3′-OH in their ribose ring. Cidofovir is a phosphonate-containing acyclic cytosine analogue that, unlike the inhibitors described above, does not depend on viral enzymes for its conversion to the triphosphorylated form that competes with the dNTP substrates [9]. Foscarnet is an analogue of pyrophosphate, a product of the nucleotide incorporation reaction, and therefore behaves as an inhibitor of DNA polymerization. Unfortunately, our understanding of the mechanisms involved in resistance to acyclovir and other related inhibitors is limited by the absence of crystal structures of herpesvirus DNA polymerases.

Table 20.1 Antiviral drugs approved for clinical use

Target	Step of the viral life cycle or cellular function inhibited	Virus	Drug type and name
Viral	Entry	HIV	Fusion inhibitors: Enfuvirtide
	Disassembly/uncoating	Influenza virus	Drugs binding to the viral protein M2 (an ion channel): Amantadine and rimantadine
	Genome replication	HIV	Nucleoside/nucleotide RT inhibitors: Zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), abacavir (ABC), emtricitabine (FTC) and tenofovir (tenofovir disoproxil fumarate)[a]
			Nonnucleoside RT inhibitors: Nevirapine, delavirdine, efavirenz, etravirine and rilpivirine
		HBV	Nucleoside/nucleotide analogues: Lamivudine, emtricitabine, entecavir, telbivudine, adefovir (adefovir dipivoxil)[a] and tenofovir (tenofovir disoproxil fumarate)[a]
		HSV and VZV	Nucleoside/nucleotide analogues: Acyclovir (valaciclovir),[a] penciclovir (famciclovir),[a] idoxuridine, trifluridine and brivudine
		HCMV	Nucleoside/nucleotide analogues: Ganciclovir (valganciclovir) and cidofovir
			Pyrophosphate analogue: Foscarnet
	Integration into the host genome	HIV	HIV integrase inhibitors: Raltegravir and elvitegravir
	Synthesis of viral mRNAs	HCMV	Antisense (phosphorothioate) oligonucleotide: Fomivirsen
	Cleavage of viral polyproteins	HIV	HIV protease inhibitors: Saquinavir, ritonavir, indinavir, nelfinavir, amprenavir and its prodrug fosamprenavir, lopinavir, atazanavir, tipranavir and darunavir
		HCV	HCV protease inhibitors: Telaprevir and boceprevir
	Budding	Influenza virus	Viral neuraminidase inhibitors: Oseltamivir and zanamivir
Cellular	Viral entry	HIV	Viral coreceptor inhibitors: Maraviroc
	Innate immunity	HCV and HBV	Interferons: Pegylated interferons α-2a and α-2b, and interferons α-2a and α-2b
	mRNA capping enzymes and viral mutagenesis	HCV and influenza virus	Ribonucleoside analogue: Ribavirin

[a]Compound approved as a pro-drug, whose name is indicated between parentheses

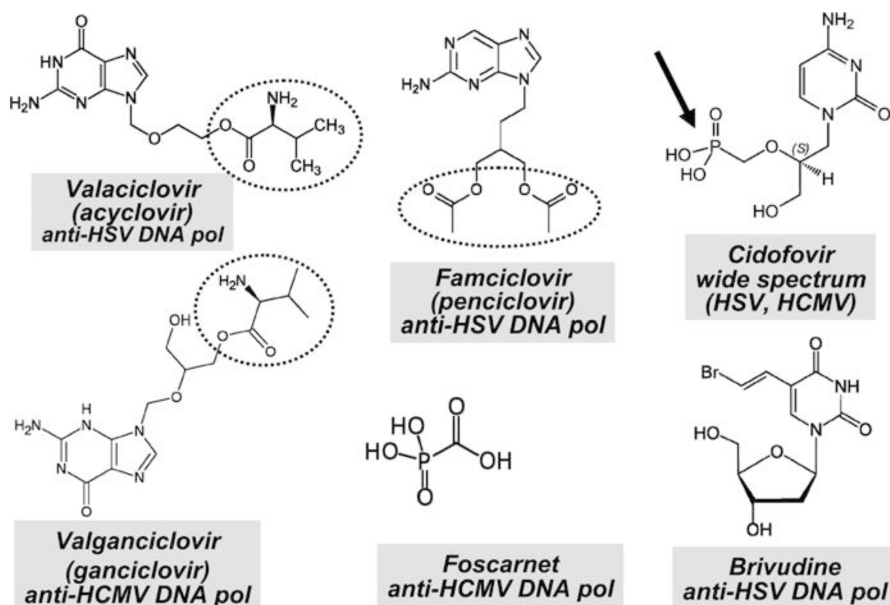

Fig. 20.3 Approved nucleoside, nucleotide and pyrophosphate analogues with antiviral effect on herpesviruses. Valaciclovir, famciclovir and valganciclovir are prodrugs of acyclovir, penciclovir and ganciclovir, respectively. Protecting groups that favor oral absorption are shown within *dotted ellipses*. *Arrow* shows the location of the phosphate group in the nucleotide analogue. *HSV* herpes simplex virus, *HCMV* human cytomegalovirus

HIV is a retrovirus that replicates through a proviral double-stranded DNA intermediate. Its polymerase, known as reverse transcriptase (RT), is able to synthesize DNA by using either RNA or DNA as templates. Nucleoside/nucleotide analogues and nonnucleoside RT inhibitors (NNRTIs) constitute the backbone of current antiretroviral therapies [2, 3]. Nucleoside/nucleotide RT inhibitors are prodrugs that need to be converted to active triphosphate analogues in order to be incorporated into the DNA during reverse transcription. They act as chain terminators thereby blocking DNA synthesis [10]. The HIV-1 RT is a heterodimer composed of subunits of 560 and 440 amino acids (known as p66 and p51, respectively). Both polypeptide chains have the same amino acid sequence, but the large subunit contains an RNase H domain that includes the 120 amino acids of its C-terminal region. The DNA polymerase domain is formed by residues 1–315 and aspartic acid residues 110, 185 and 186 in p66 constitute the catalytic triad.

There are many crystal structures available for HIV-1 RT, including relevant complexes such as the ternary complex of HIV-1 RT/double-stranded DNA/dTTP, or RT bound to RNA/DNA or to DNA/DNA template-primers. Additional structures containing DNA primers blocked with nucleoside analogues have also been described. A large number of NNRTIs have been crystallized in complex with the viral polymerase (for a review, see [11]). NNRTIs bind to a hydrophobic pocket

located about 8–10 Å away from the DNA polymerase active site. The first NNRTIs were identified by using an antiviral screening approach, but structure-based drug design has played a prominent role in the design and development of next-generation NNRTIs such as etravirine or rilpivirine [12, 13].

Reverse transcription is also involved in the replication of HBV. These viruses form an immature RNA-containing nucleocapsid that, inside the cell, is converted into a mature nucleocapsid containing a relaxed circular DNA [14]. The DNA found in extracellular virions is synthesized by viral RT using the pregenomic RNA as a template. The HBV RT shows sequence similarity with HIV-1 RT, and several inhibitors of HIV-1 replication (*e.g.* lamivudine, adefovir or tenofovir) have been approved from treating HBV infections (Table 20.1). Unfortunately, there is no available crystal structure for the HBV RT. The existence of this gap of knowledge can be attributed to major difficulties in obtaining a catalytically active polymerase.

RNA polymerases play a key role in the replication of important pathogenic RNA viruses such as HCV, poliovirus, rhinovirus, influenza virus and others. However, drugs targeting viral RNA polymerases have not received approval. At present, HCV RNA polymerase inhibitors are probably the ones in a more advanced stage of development [15, 16]. The HCV replicase (NS5B, an RNA-dependent RNA polymerase) is a 65-kDa protein that contains a C-terminal membrane insertion sequence that traverses the phospholipid bilayer as a transmembrane segment. NS5B inhibitors can be classified into nucleoside and nonnucleoside analogues. Examples of the first group are valopicitabine, 2′-*C*-methyladenosine, 4′-azidocytidine and mericitabine. The nucleotide prodrug β-D-2′-deoxy-2′-α-fluoro-2′-β-C-methyluridine (PSI-7977) [17] is currently in phase III clinical trials and showed promising results when combined with pegylated interferon and ribavirin. Nonnucleoside inhibitors of NS5B are tegobuvir, filibuvir and pyranoindoles such as HCV-371 and HCV-570 [18].

The non-structural 5A (NS5A) protein of HCV has been identified as the target of daclatasvir (BMS-790052), a thiazolidinone-containing small molecule that was shown to inhibit HCV replication in a cell-based replicon screen [19]. The inhibitor showed picomolar potency in preclinical assays. The enzymatic function of NS5A is not known, although it interacts with NS5B and modulates the host cell interferon response. Resistance to daclatasvir is associated with mutations in the N-terminal region of NS5A [20]. Interestingly, the cyclic endecapeptide alisporivir, currently in Phase III clinical trials, is a cyclophilin A-binding drug that selects for mutations in NS5A, although in a different domain [21].

Drugs Interacting with Other Targets

Gene expression is another possible target for antiviral intervention. A phosphorothioate derivative of the oligonucleotide 5′-GCG TTT GCT CTT CTT CTT GCG-3′ (known as fomivirsen) has been used as a repressor of the synthesis of early genes in HCMV infections. It binds to the complementary sequence in the viral mRNA, blocking its translation. In retroviruses, integration of the double-stranded proviral DNA (the product of reverse transcription) into the host cell DNA is essential for expression of viral mRNA. Retroviral mRNA is generated by transcription of the integrated provirus by cellular RNA polymerases.

Raltegravir and elvitegravir are inhibitors of HIV-1 integrase [22, 23], an enzyme endowed with 3′ endonucleolytic and strand transfer activities. It is composed of three structural domains: an N-terminal domain of unknown function, the central catalytic domain, and a C-terminal domain with DNA binding activity. Approved drugs bind to the central domain, close to the catalytic residues Asp64, Asp116 and Glu152, and act by blocking the strand transfer activity of the integrase. Crystal structures of catalytic domains have been used in the design of integrase inhibitors, but the full integrase (having all three domains) has become available only very recently [24].

Viral proteins are frequently synthesized as precursor polypeptides that are cleaved by viral proteases. Sometimes, this cleavage occurs before the assembly of the viral capsid, which is formed by the association of mature proteins into capsomers. For example, in poliovirus, rhinovirus and other picornaviruses, 60 protomers made of processed capsid proteins VP0, VP1 and VP3 assemble to form the icosahedral particle. In HCV, its serine protease (designated as NS3) interacts with an NS4A peptidic co-factor that modulates its proteolytic activity. Promising NS3/4A inhibitors were developed after rational structure-based design using a hexapeptide (Asp-Asp-Ile-Val-Pro-Cys) derived from the natural NS5A/5B cleavage site as a lead compound. After systematic replacement of single amino acid residues, important substituents in the protease binding pocket were identified and docked in the crystal structure of the unliganded NS3 protease. Further optimization led to the identification of ciluprevir (BILN-2061), one of the first promising drug candidates (Fig. 20.4). The recently licensed inhibitors boceprevir [25] and telaprevir [26] were developed by using similar approaches.

20.3.2 Inhibitors of Viral Assembly

Despite considerable efforts in recent years, drugs targeting the assembly of viral capsids (see Chaps. 10 and 11) have not yet gained approval for clinical use. At preclinical stages of development, there are interesting examples of drugs inhibiting the assembly of capsids of HBV (*e.g.* heteroaryldihydropyrimidines) [27, 28] and HIV-1 (CAI, CAP-1, bevirimat, etc....) [29, 30] (see Sects. 20.6.2 and 20.6.3).

20.3.3 Budding Inhibitors

At present, antiviral treatment and prophylaxis of human influenza infections, including aggressive strains such as avian H_5N_1, are based on the use of oseltamivir and zanamivir that inhibit the release of infectious virus. The influenza virus membrane contains a glycoprotein (hemagglutinin) that recognizes sialic acid in cell-surface glycoproteins and glycolipids [31]. Sialic acid is the receptor for virus entry. However, newly formed virions may remain attached to the cell membrane

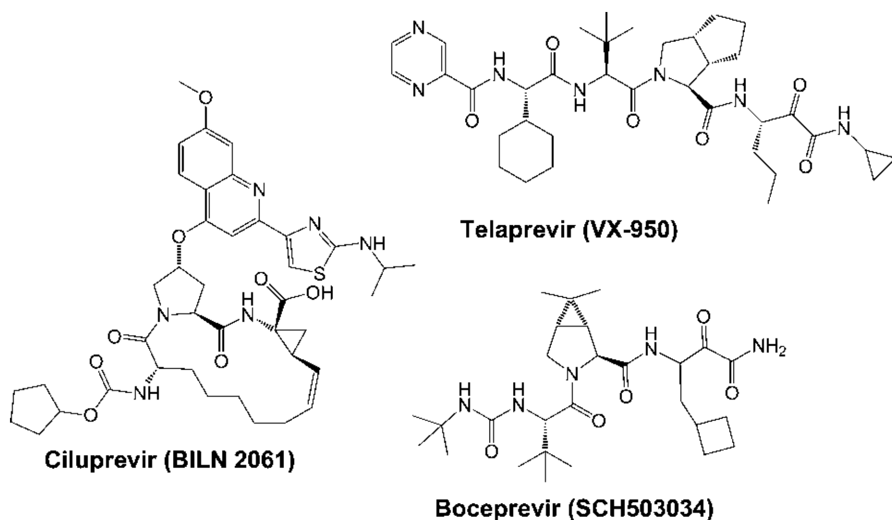

Fig. 20.4 Hepatitis C virus protease inhibitors

because of the interaction between hemagglutinin and sialic acid. The viral neuraminidase (also known as sialidase) facilitates the release of virions from infected cells by cleaving sialic acid residues. Oseltamivir and zanamivir inhibit this hydrolytic activity.

20.3.4 Maturation Inhibitors

Maturation is a relatively common process in animal and human viruses (see Chap. 13). In contrast to HCV and other virus families, specific cleavage of the polypeptides encoding the mature viral proteins occurs only on assembled immature particles. HIV-1 protease inhibitors block the proteolytic processing of precursor polypeptides Gag and Gag-Pol. Cleavage of Gag and Gag-Pol leads to the formation of structural proteins (*e.g.* MA, CA and NC), p6 and the viral enzymes protease, RT and integrase (Fig. 20.5). This process occurs extracellularly and is required for the formation of an infectious virion. The HIV-1 protease is a homodimeric enzyme composed of subunits of 99 amino acids with a symmetric substrate binding pocket. Inhibitors were initially designed as peptide derivatives mimicking the 'transition-state' and contained a non-hydrolysable bond at the position where the cleavage was expected to occur. All of the approved HIV protease inhibitors, except tipranavir, can be considered as peptidomimetics [2].

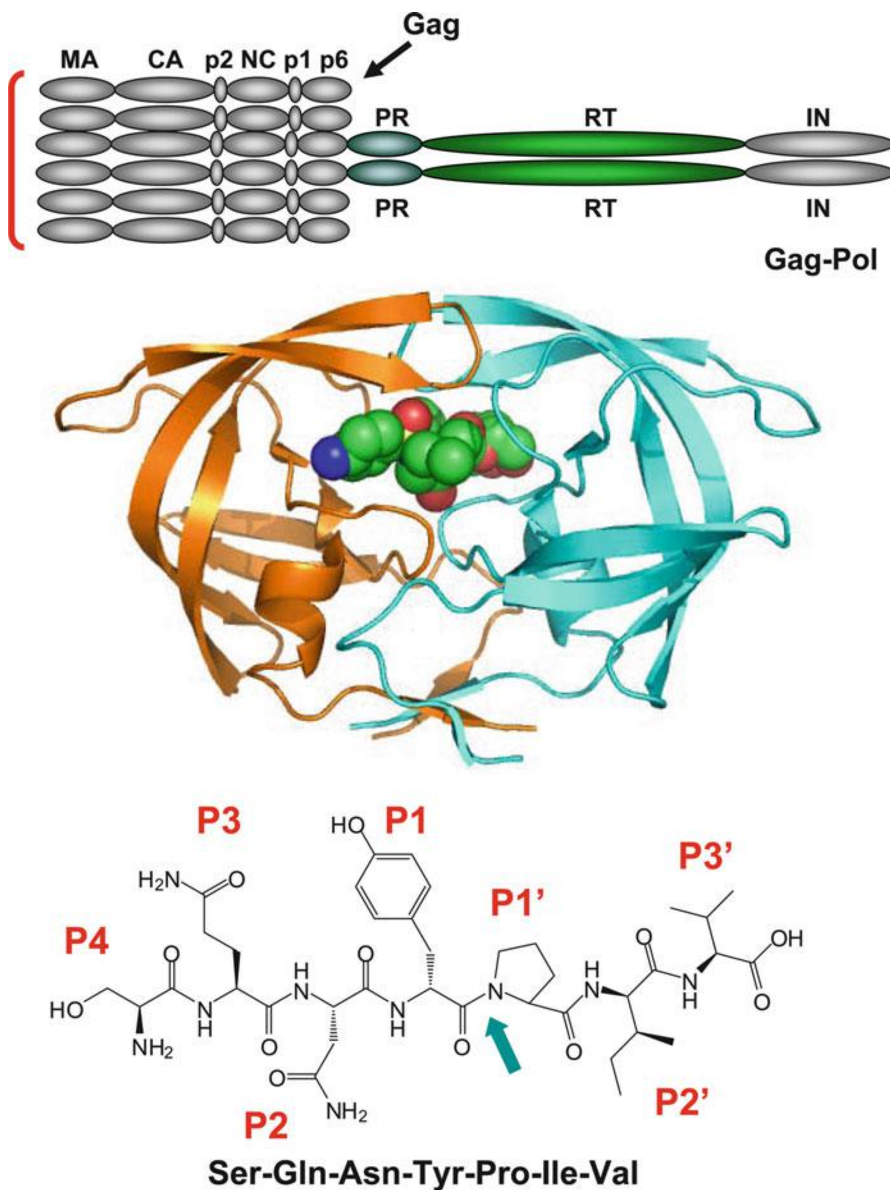

Fig. 20.5 Protein arrangement in Gag and Gag-Pol precursor polyproteins, HIV-1 protease structure and model substrate. Cartoon representation of the protease bound to darunavir (PDB entry 1T3R). Inhibitor C, O and N atoms are displayed as *green*, *red* and *purple spheres*, respectively. The minimal peptide substrate SQNY*PIV (asterisk indicates cleavage site) is shown below the protease structure

20.3.5 Drugs Targeting Viral Entry

Drugs acting at this step include compounds that block cell attachment, receptor recognition or the mechanisms leading to the translocation of the viral capsid to the host cell cytoplasm (see Chaps. 15 and 16). Two anti-HIV drugs belonging to this category have been approved: maraviroc and enfuvirtide. The first interferes with coreceptor recognition, while the second blocks the fusion of the viral envelope with the cell membrane.

Receptor and Coreceptor Recognition in HIV-1 Infection

HIV-1 enters target cells through a multi-step process that requires non-specific interactions with surface heparan sulfates, followed by binding of the viral envelope glycoprotein gp120 to its primary cellular receptor CD4. Attachment inhibitors in HIV-1 include dextran sulphate and other polyanions, as well as proteins binding glycosidic residues (*e.g.* mannose-specific lectins or cyanovirin N).

CD4 binds into a carbohydrate-free depression in gp120 that is poorly accessible to immunoglobulins. However, crystallographic studies carried out with complexes of gp120, CD4 and Fab fragments of neutralizing antibodies have shown that Phe43 of CD4 and residues 365–371 and 425–430 of gp120 make the largest contributions to interatomic contacts [32]. Phe43 is located on the CDR2-like loop of CD4 and binds within a hydrophobic pocket of gp120, termed the "Phe43 cavity", while Arg59 (in CD4) is located on a neighboring β-strand and forms an ion pair with the gp120 residue Asp368. The disruption of this interaction by small molecule binding has been validated as a promising approach to prevent HIV-1 entry. Inhibitors containing a tetramethylpiperidine ring such as NBD-556 displayed remarkable antiviral activity and altered the gp120 conformation in a similar way as observed upon CD4 binding [33, 34].

CCR5 and CXCR4 are the necessary coreceptors for cellular entry of HIV-1. These molecules are members of the seven-transmembrane G protein-coupled receptor superfamily, and can be blocked by natural ligands such as MIP-1α, MIP-1β and RANTES for CCR5 and SDF-1 for CXCR4. RANTES derivatives and SDF-1α have been investigated as potential inhibitors of viral entry. However, smaller CCR5 antagonists such as TAK-652 and TAK-779, aplaviroc, vicriviroc and maraviroc were further studied as potential anti-HIV agents. So far, maraviroc remains as the only clinically approved coreceptor antagonist. However, resistance to this drug appears mostly as a result of HIV-1 using CXCR4 as an alternate coreceptor for entry [3].

The most advanced CXCR4 antagonist, AMD3100 (Mozobil) has not been further studied as a therapeutic agent against HIV-1 [18]. However, in combination with granulocyte colony stimulating factor (and under the name of plerixafor), it has been approved for the mobilization of hematopoietic stem cells to the peripheral blood for collection and subsequent autologous transplantation in patients with non-Hodgkin's lymphoma and multiple myeloma.

Fusion Inhibitors: Targeting the Formation of Coiled-Coil Structures

Viral recognition by cellular receptors triggers a mechanism that leads to the fusion of the viral envelope and the target cell membrane [35] (see Chap. 16). Crystallographic studies of pre- and post-fusion conformations of the influenza virus hemagglutinin showed that the formation of a six-helix bundle played a major role in the fusogenic events. Drugs targeting the helix bundle have been developed to block entry of HIV-1 and respiratory syncytial virus (RSV).

In the case of HIV-1, enfuvirtide is a 36-amino acid peptide that derives from residues 127–162 of the transmembrane protein gp41. This glycoprotein has a trimeric structure, and each monomer contains a transmembrane region of 21 residues, located between an N-terminal ectodomain (175 residues) and a long cytoplasmic domain of 150 amino acids. The sequence of enfuvirtide overlaps with the heptad repeat two (HR2) region (residues 117–154) that interacts with the HR1 region (residues 29–82) of gp41. Enfuvirtide binds HR1 and prevents the formation of the trimeric coiled-coil structure required for membrane fusion. Despite showing efficacy *in vivo*, the clinical use of the drug has been limited by its reduced bioavailability and its large production costs. Novel anti-HIV drugs based on similar principles include tifuvirtide, sifuvirtide and T-2635. These peptides mimic in part the HR2 structure and exert their effects through a mechanism of action similar to that shown by enfuvirtide [35].

In the case of the human RSV, an orally active candidate (BMS-433771) was proposed to bind in a hydrophobic cavity within the trimeric N-terminal heptad repeat (equivalent to HR1), which is the region that associates with a C-terminal HR2 to form a six-helical coiled-coil bundle during the fusion process. X-ray crystallography of another drug candidate (TMC353121) bound to the human RSV fusion protein (Protein Data Bank (PDB) entry 3KPE) showed that this drug, rather than preventing formation of the coiled-coil, produces a local disturbance of the six-helix bundle by stabilizing the HR1 – HR2 interactions in an alternate conformation of the six-helix bundle [36].

20.3.6 Uncoating Inhibitors

Amantadine and rimantadine were used more than 20 years ago to prevent influenza A virus infections. However, they never gained wide acceptance and today virtually all strains are resistant to these agents. These compounds block the release of the viral capsid to the cytoplasm of the infected cell, because they interact with M2, an integral protein located in the viral envelope. It consists of four identical units, each one having three distinct domains: (i) an ectodomain made up of 24 residues exposed to the outside environment, (ii) a transmembrane region (mostly hydrophobic) composed of 22 amino acids, and (iii) 52 amino acids at the C-terminal region exposed to the intraviral milieu. M2 is activated by low pH and functions as an ion channel that facilitates entry of protons into the viral particle. Amantadine and rimantadine bind to the transmembrane region (Fig. 20.6), sterically blocking the channel and inhibiting the release of the viral capsid to the host cell cytoplasm.

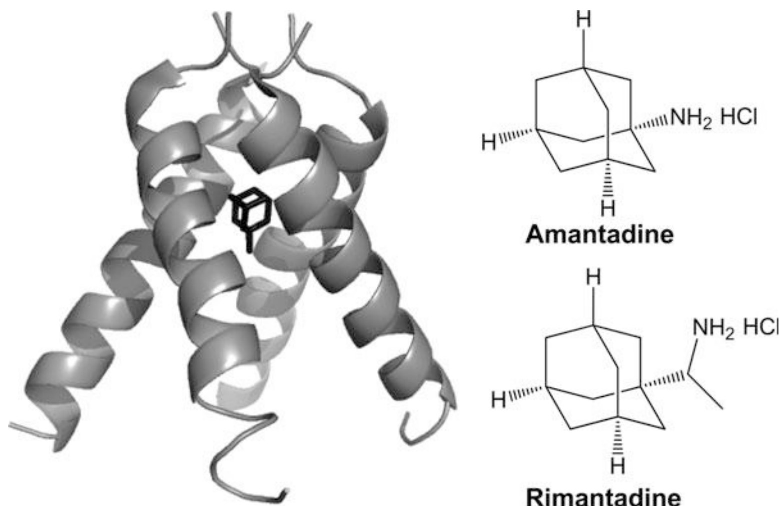

Fig. 20.6 Transmembrane region of influenza virus M2 protein bound to amantadine and chemical structures of drugs blocking the release of the viral capsid into the cytoplasm. Cartoon representation of the transmembrane regions of the M2 tetramer and stick model of the bound inhibitor (coordinates were taken from PDB entry 3C9J)

20.4 Strategies in the Development of Antiviral Drugs: From Random Screening to Structure-Based Design

Most of the antiviral drugs clinically used today and mentioned in previous sections of this chapter were discovered serendipitously or as a result of screening campaigns that made use of large libraries of synthetic compounds or natural products. In the traditional drug discovery process, it is estimated that screening of around 80,000 molecules can render one hit that needs to be further optimized to a lead compound by improving its activity and reducing its toxicity before entering clinical trials. Usually, these huge efforts are needed because of the lack of structures for ligands and protein targets. In this scenario, pharmaceutical companies invested in the development of high-throughput screening (HTS) assays and in the generation of large chemical libraries obtained by combinatorial chemistry. Nevertheless, this process is expensive, time-consuming and requires further efforts in target validation.

Random approaches including HTS and combinatorial chemistry are used in the absence of structural information about ligands or target proteins or macromolecular complexes. Over the last two decades we have been able to witness many profound changes in the drug discovery paradigm due to major advances in such diverse areas as HTS methods, genome sequence projects, macromolecular structure determination, quality and scope of publicly accessible databases, computer performance and new algorithms for '*in silico*' approaches [37] (Fig. 20.7). Among the latter, structure-based and ligand-based virtual screening (SBVS and LBVS, respectively) stand out as powerful tools that are being increasingly used in both

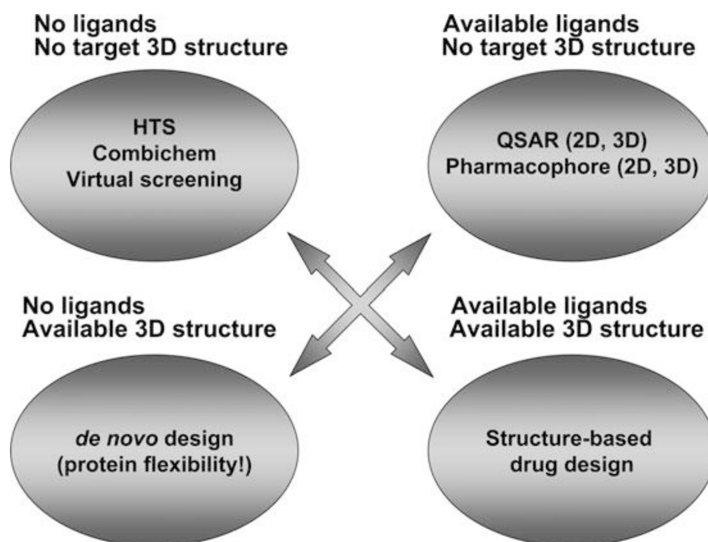

Fig. 20.7 Strategies in drug design depending on the availability of structural information for ligands and biological targets. Abbreviations: *2D* bidimensional, *3D* three-dimensional, *HTS* high-throughput screening, *QSAR* quantitative structure-activity relationship

industry and academia [38]. These techniques rely on the three-dimensional coordinates of either the macromolecular target (SBVS) or one or more known ligands (LBVS) to search in chemical or fragment libraries for putative hits that can then be tested experimentally for confirmation of affinity/activity and then eventually transformed into lead compounds. Due to the huge amount of ligands that can be found in currently available databases (*e.g.* 13 million in ZINC [39]), it is customary to employ a series of computational cost-effective filters to narrow down the number of molecules that will be subjected to virtual screening. Lipinski's rule of five[1] [40] and/or other physico-chemical-based principles can be used for filtering, as well as pharmacophoric hypotheses. One goal of LBVS is to find similarly shaped molecules that belong to novel chemotypes ("scaffold hopping") [41].

20.4.1 Structure-Based Drug Design

Structure-based drug design relies on knowledge of the three-dimensional structure of the biological target gained by means of techniques such as X-ray

[1] Lipinski's rule states that, in general, an orally active drug should meet at least three of the following criteria: (i) Not more than five hydrogen bond donors (nitrogen or oxygen atoms with one or more hydrogen atoms), (ii) not more than ten hydrogen bond acceptors (nitrogen or oxygen atoms), (iii) a molecular mass <500 daltons, and (iv) an octanol-water partition coefficient log P not greater than five.

crystallography (Chap. 4), nuclear magnetic resonance (NMR) spectroscopy (Chap. 5) and electron microscopy (Chap. 3). A number of computational methods have been developed to identify putative ligands that can fit into an active site or functional epitopes in the target protein or molecular complex. Success in molecular docking, which may be defined as an optimization problem that describes the "best-fit" orientation of a ligand within a binding site [42], very often depends on the quality of the three-dimensional target protein structure. Structures determined by X-ray crystallography at atomic or near-atomic resolution are the most adequate for docking. More specifically, successful identification of lead compounds usually requires a crystallographic resolution below 2.5 Å and an R factor well below 25 % (see Chap. 4).

20.4.2 Ligand Docking and Virtual Screening

Docking methods are calibrated and tested for their ability to predict, at the top of the list of possible solutions, the native conformation of a ligand bound to a protein as found in a representative series of crystallographic complexes. To assess the performance of a docking program in SBVS its ability to discriminate true binders from a pool of fake ligands ("decoys") is also examined. The goodness of fit between the ligand and the receptor is evaluated by means of a scoring function composed of different terms that attempt to account for the forces driving the binding event. Although the underlying physical laws describing the association process are well understood, accuracy and computational resources (mainly execution time) evolve in opposite directions, and fine tuning the appropriate balance between them is not an easy task. Therefore, accuracy is normally sacrificed for speed, especially in SBVS where very often too simplistic scoring functions are employed which plainly cannot capture the true free energy of binding that is measured experimentally [43]. Ligand conformers can be generated within the binding site itself "on the fly" for each target, or created beforehand as a collection of all major theoretically possible conformers following some pre-defined rules. The former method is more computer-intensive but, as an advantage, it can generate strained conformations that adapt better to each specific binding site environment. The latter method allows the stored coordinates to be reused again as many times as needed in different SBVS campaigns but the drawback is that a relevant conformation that deviates slightly from a canonical one can be missed.

Fragments [molecular weight $\leq$ 250 Da, log P $\leq$ 2.5, (where P refers to the octanol-water partition coefficient) and less than 6 rotatable bonds] are usually endowed with reduced affinities compared to conventional ligands but are better suited for chemical modifications aimed at producing novel drug candidates [44]. They can sample chemical space more effectively than do regular ligands. It is generally accepted that fragment docking outperforms traditional small-molecule docking in terms of hit rates even though erroneous binding modes can be obtained for them because their volumes are much smaller than those of typical binding site

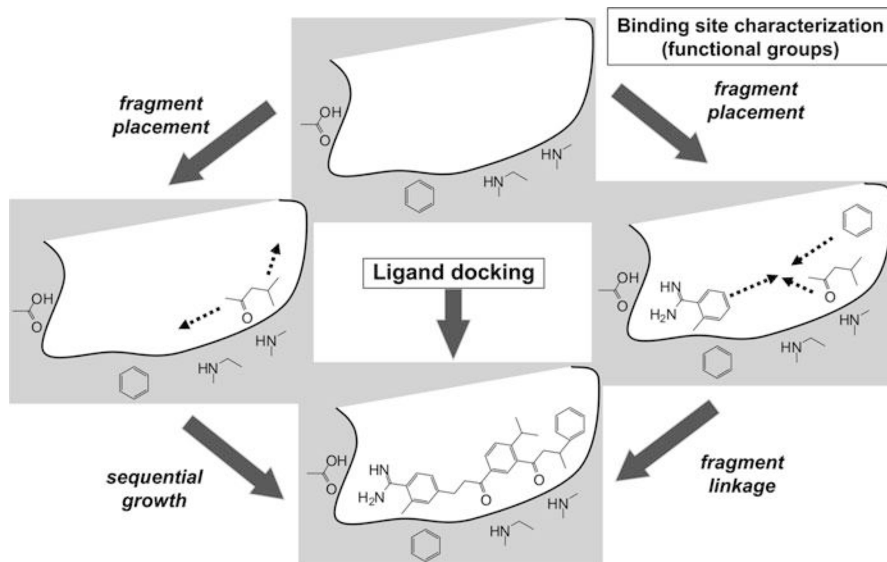

Fig. 20.8 Schematic diagram showing ligand- and fragment-based virtual screening strategies

cavities (Fig. 20.8). Docked fragments can then evolve virtually (by adding different chemical decorations), be linked (to join two or more fragments that occupy different regions of the binding site), self-assemble (through direct bond formation between different reacting fragments), and/or be optimized to better fulfill drug-like properties. Since the binding affinity of compounds has been shown to grow approximately linearly with their molecular weight, the affinity of fragments and standard drugs are usually compared in terms of a metric called ligand efficiency index, which is defined as the binding free energy per heavy atom. Given that affinity/activity information related to ligand-target complexes is currently stored in several databases (*e.g.* BindingDB, ChEMBL, and PDBBind, among others), ligand efficiency indices have been proposed as useful variables to chart the chemico-biological space and visualize it in an atlas-like type of representation [45].

The binding pockets that are explored in most SBVS campaigns are relatively small cavities that are meant for natural agonists, substrates or cofactors in a limited number of macromolecular targets. These represent what are known as the “orthosteric” binding sites for drugs. However, other drugs (*e.g.* NNRTIs) exert their action by altering the receptor’s conformation in such a way that it would affect the binding and/or functional properties of agonists or substrates (“allosteric modulators”) whereas yet others are known to modulate or prevent some key protein-protein interactions.

Most protein-protein interfaces have been usually regarded as “undruggable” because conventional analysis reveals them as lacking well-defined binding pockets. However, it has been noted that a large percentage of the free energy of interaction arises from small regions designated as “hot spots”. These facts supported the concept by which protein-protein interaction disruptors do not need

to mimic the entire protein binding surface but rather a smaller subset of key residues in a relatively compact region of one of the binding partners.

Different computational tools have been developed for exploring the properties of protein-protein interfaces and facilitate the identification of starting points for small-molecule design (*e.g*. PocketQuery [46], http://pocketquery.csb.pitt.edu/). More generally, "druggable" sites can be located using FTMap [47], which is an efficient fast Fourier transform correlation method that samples multiple positions of up to 16 different probes on a three-dimensional grid centered on a protein of interest. A simple energy expression then allows the identification of consensus sites where probes tend to cluster, giving rise to favorable interactions. These sites correlate with locations where high-affinity ligands are found in protein complexes. The method is sensitive to conformational changes in the protein and can be used to map affinity sites prior to docking. The FTMap algorithm is implemented in the public domain Web server http://ftmap.bu.edu/.

20.4.3 Structure-Activity Relationships

Finally, visual examination of the crystal structure of a single ligand-receptor complex is usually highly informative and can guide the design of new analogues that will display graded affinities toward the target. However, a quantitative assessment of these variations will generally be difficult because the observed free energy differences result from a subtle interplay of binding forces within the receptor binding site that normally take place in the face of competition with water molecules. An approach that has been shown to be useful in these cases is to model the whole set of complexes and decompose the ligand-receptor interaction energies into a number of van der Waals and electrostatic contributions emanating from individual receptor amino acids, together with additional terms representing the desolvation of both binding partners. The resulting matrix of energy terms can then be projected onto a small number of orthogonal "latent variables" or principal components using partial least squares in a way similar to that used in comparative molecular field analysis (CoMFA). At the end of the procedure, those pair-wise interactions between the ligands and individual protein residues that are predictive of binding affinity or activity are selected and weighted according to their importance in the model. Since its inception, this chemometric method, termed comparative binding energy (COMBINE) analysis, has been successfully applied to a variety of biologically relevant targets including the protease and the RT of HIV-1 [48].

20.5 Case Studies in Structure-Based Antiviral Drug Development

During the last three decades we have witnessed an unprecedented effort to discover new antiviral drugs, most of them directed towards HIV, and more recently towards HCV. Many of those studies have benefited from the availability

of high-resolution crystal structures. We have selected a few case studies to illustrate how structure-based design has contributed to antiviral drug discovery. In this section we describe two successful examples that include oseltamivir and zanamivir acting on influenza virus neuraminidase, and mutation-resilient inhibitors of HIV-1 protease (*e.g.* darunavir). In the next section (20.6) we describe current experimental efforts in structure-based design, applied to inhibitors acting on the assembly or disassembly of viral capsids.

20.5.1 Neuraminidase Inhibitors Against the Threat of Influenza Pandemics

Although the crystal structure of influenza virus neuraminidase (also known as sialidase) was determined in 1983 [49], its resolution was relatively low (approx. 3.0 Å) and therefore, of limited value in structure-based drug design. Initial efforts to design neuraminidase inhibitors were based on substrate-like compounds such as *N*-acetylneuraminic acid (Neu5Ac) derivatives (Fig. 20.9). One of them, the 2-deoxy-α-Neu5Ac demonstrated good activity in mouse model experiments. The improvement in resolution of the neuraminidase X-ray structures obtained in complex with Neu5Ac or with the derivative Neu5Ac2en facilitated the design of more potent inhibitors. These studies revealed that sialic acid adopts a quite deformed conformation when bound to neuraminidase, due to strong ionic interactions of the sialic acid carboxyl moiety with three arginine residues of the enzyme (Arg118, Arg192 and Arg371). Starting with Neu5Ac2en, the substitution of the C-4 hydroxyl group with a guanidinyl functionality rendered the 4-deoxy-4-guanidino-Neu5Ac2en (zanamivir) that showed significantly improved affinity promoted by interactions between the guanidinyl moiety and the neuraminidase residues Glu119 and Glu227 [50]. Zanamivir was found to be highly selective for influenza virus sialidase. It was approved in 1999 as the first neuraminidase-targeting anti-influenza drug (Fig. 20.9) even though it can be administered only as a powder for oral inhalation.

Oseltamivir was developed as a result of efforts to develop neuraminidase inhibitors based on non-carbohydrate templates [50]. In this context, a cyclohexene ring was considered as a transition-state mimic, and amenable to chemical modifications to optimize its biological activity. X-ray crystallographic studies of sialic acid and related analogues bound to neuraminidase (Fig. 20.9) demonstrated that the C7 hydroxyl group of the glycerol side chain does not interact with amino acid residues in the sialidase active site. Replacement of the glycerol moiety to improve the lipophilicity of those inhibitors was carried out even though the hydroxyl groups at C8 and C9 were involved in interactions with Glu276 and Arg224. A 3-pentyl ether side chain at position C7 gave optimal results and led to the development of oseltamivir. Oseltamivir (originally known as GS 4104) is an ethyl ester prodrug of GS 4071 that can be administered orally as capsules or as a

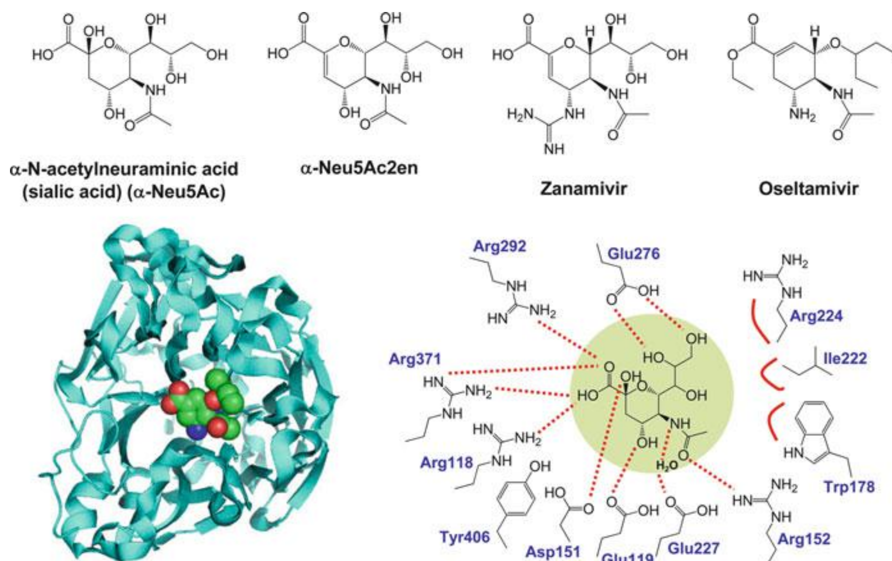

Fig. 20.9 Influenza virus neuraminidase inhibitors and structural basis of their mechanism of action. The lower part shows the structure of a neuraminidase monomer with a molecule of GS 4071 (the oseltamivir prodrug). C, O and N atoms in the inhibitor are shown in *green*, *red* and *purple*, respectively (shown structure was taken from the PDB file 2HT8). The scheme on the right shows hydrogen bonding network (*red dotted lines*) and hydrophobic pockets (*continuous red line*) in the sialic acid binding site of influenza virus neuraminidase (Based on the scheme published in Ref. [50])

suspension, and is then converted to the active form *in vivo* by the action of endogenous esterases. Crystal structures of influenza virus neuraminidase bound to GS 4071 showed a rearrangement of the side-chain of Glu276 that now interacts with Arg228, generating a larger hydrophobic area in this domain. The amino acid substitution H274Y decreases neuraminidase binding affinity for oseltamivir and is the major mutation involved in resistance to this antiviral drug. Crystal structures of oseltamivir-resistant mutant neuraminidases have shown that the presence of Tyr instead of His274 affects the positioning of Glu276 and disrupts the hydrophobic pocket that accommodates the 3-pentyl ether side chain of oseltamivir [35, 51].

20.5.2 Development of Darunavir to Combat Resistance to HIV-1 Protease Inhibitors

The HIV-1 protease is a homodimer that contains two catalytic aspartic acid residues (one in each subunit) in the active site (Fig. 20.5). The Asp25 residues share an acidic proton and interact with a water molecule in the absence of substrate or inhibitor. The hydrolysis mechanism involves activation of a water molecule by these aspartates and nucleophilic attack of the water oxygen to the amide carbonyl

of the bond to be cleaved between P1 and P1′. Breakdown of the resulting tetrahedral intermediate by successive proton transfers leads to the amino and carboxylate products. The scissile bond of the peptide substrate is in close proximity of the active site. Upon binding, flexible flaps (one per monomer) that adopt an open conformation in the apoenzyme fold down over the substrates. The peptide substrates contain at least seven residues extending from P4 to P3′, where the cleavage site lies between P1 and P1′. The side chains of the substrate lie in the protease subsites S4 to S3′. Replacement of the hydrolyzable peptide linkage by a non-cleavable bond was a basic principle for the development of peptidomimetic inhibitors that culminated with the approval of saquinavir [52].

Saquinavir and other related protease inhibitors have serious drawbacks that include severe side effects and toxicities, poor bioavailability (high doses required to maintain their therapeutic effect), costly synthesis and the rapid emergence of drug resistance. Extensive analysis of protein-ligand X-ray structures of wild-type and mutant HIV-1 proteases showed that there were minimal alterations in the active site backbone conformation of proteases resistant to inhibitors. Therefore, drugs effective on resistant HIV-1 strains should maintain extensive hydrogen-bonding interactions with the protein backbone of the wild-type enzyme.

The replacement of the asparagine moiety at the P2 position in saquinavir with 3*R*-tetrahydrofuranyl glycine resulted in a more potent inhibitor (Fig. 20.10). Other studies leading to the development of fosamprenavir/amprenavir showed an improvement in antiviral activity by introducing the 3*R*-tetrahydrofuranyl urethane moiety onto a hydroxyethylamine sulfonamide isostere. However, crystal structures revealed that 3*R*-tetrahydrofuranyl urethane-bearing inhibitors had weak hydrogen bonding potential with main chain atoms of residues Asp29 and Asp30. Therefore, a bicyclic tetrahydrofuran (bis-THF) ligand was designed and developed to improve hydrogen bonding with both aspartic acid residues. Inhibitors incorporating a P2 bis-THF and a *p*-methoxysulfonamide as the P2′ ligand (*e.g.* TMC-126) were remarkably potent in enzyme inhibition assays and displayed strong antiviral activity in cell culture. Darunavir was similar to TMC-126 but contained *p*-aminosulfonamide instead of *p*-methoxysulfonamide at the P2′ position. The P2′-amine group provided more favorable pharmacokinetic properties compared to the P2′-methoxy group. Darunavir-bound X-ray crystal structures showed that both P2 and P2′ moieties are involved in extensive hydrogen bonding with the protein backbone. These tight interactions were consistently observed with mutant proteases and might be responsible for the unusually high resistance profile of darunavir. Detailed reviews describing the design and development of darunavir have been published [53].

20.6 Viral Capsids as Targets of Antiviral Intervention

Remarkable examples of structure-based design of preclinical drugs binding to the mature or immature viral capsid or interfering with their assembly or conformational transitions required for genome uncoating have been described for picornaviruses, HBV and, more extensively, for HIV-1.

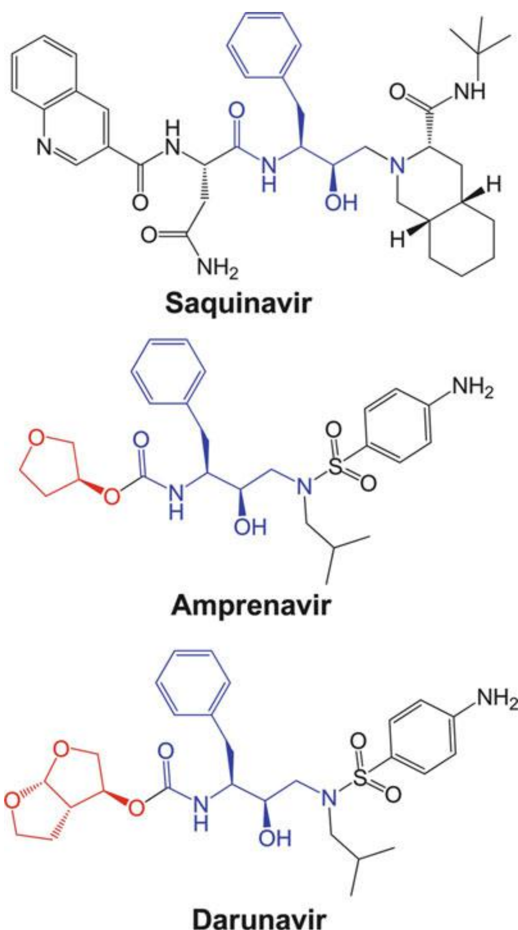

Fig. 20.10 Chemical structures of saquinavir, amprenavir and darunavir

20.6.1 Picornaviruses

The *Picornaviridae* family includes enteroviruses and rhinoviruses which are important human pathogens. Picornaviruses are non-enveloped, positive-stranded RNA viruses with an icosahedral capsid. The availability of crystal structures of poliovirus and rhinovirus (see Chaps. 4 and 10) led to the discovery of compounds that could bind to a hydrophobic pocket in the capsid protein VP1. Those molecules, known as WIN compounds (for Winthrop, the firm that developed the drugs), mimic natural factors that bind to the VP1 pocket and stabilize the capsid in a metastable conformation. During the course of a normal infection by poliovirus or rhinovirus, virion binding to the cell receptor allows for the displacement of the pocket-bound natural factors. This event triggers a global rearrangement of the capsid that is required for the release of the viral genome into the host cell cytoplasm [54]. WIN compounds and other related drugs bind to the pocket with

higher affinity than the natural factors and interfere with the receptor-induced conformational change by preventing either receptor binding or the receptor-induced rearrangement that is required for genome uncoating. In a long series of trend-setting studies, structure-function relationship analyses based on the determination of crystal structures of capsid-bound compounds, docking studies, genetic modifications of the virus particles and chemical modifications of the antiviral compounds led to the development of derivatives with improved antiviral activity.

These derivatives included pleconaril (WIN-63843) for the treatment of rhinovirus-induced exacerbation of pre-existing asthma or chronic obstructive pulmonary disease [55], BTA-798 for the enterovirus-induced sepsis syndrome [56], and V-073 with potential applications in the treatment of poliovirus infections [57]. Pleconaril binds into the hydrophobic pocket and stabilizes the metastable capsid conformation in a way that results in inhibition of both viral adsorption to cell membranes and RNA uncoating [54]. Natural resistance to this drug is largely confined to VP1 residue 191, which is valine in the vast majority of susceptible viruses and leucine in all resistant serotypes. Full resistance is achieved by an additional Tyr152 to Phe mutation [58].

20.6.2 Hepatitis B Virus

In HBV, the capsid is composed of 120 copies of a dimeric structural protein (known as CA protein) that assemble around an RNA-reverse transcriptase complex that will transcribe the RNA pregenome to the mature DNA genome. Ten years ago the fluorescent probe 5,5′-bis[8-(phenylamino)-1-naphtalenesulfonate] was shown to disturb the geometry of intersubunit contacts and to act both as a noncompetitive inhibitor of capsid assembly in HBV and as an assembly 'misdirector' that facilitates the formation of noncapsid polymers [59]. Assembly misdirection has also been shown to occur upon exposure to activator heteroaryldihydropyrimidines such as HAP1 [28, 60] and BAY 41–4109 [61].

20.6.3 Human Immunodeficiency Virus

In the case of HIV-1, we have discussed above the role of the viral protease in the release of the capsid protein (CA) from the precursor Gag polyprotein, and how protease inhibitors can prevent the formation of the mature capsid. Another compound that interferes with the maturation process is bevirimat (3-*O*-(3′,3′-dimethylsuccinyl)betulinic acid, also known as PA-457). This compound is a derivative of betulinic acid and interferes with the processing of the Gag polyprotein at the CA-SP1 cleavage site (*i.e.* between the C-terminal of the capsid protein and a spacer peptide located between CA and the nucleocapsid protein). This inhibitory action is independent of the protease activity. Tomographic reconstructions suggest

that the CA-SP1 of Gag forms a helical bundle that serves to anchor the carboxy-terminal domain (CTD) of CA below the plane of the N-terminal domain (NTD). In the absence of CA-SP1 cleavage, mature cores cannot be formed because the immature particles cannot disassemble (for reviews, see [30, 62]). Virions produced from bevirimat-treated cells display aberrant core morphologies. A similar mechanism of action has been recently described for a pyridine-based compound (PF-46396) whose inhibitory activity results in the accumulation of CA/SP1 (p25) precursor proteins and the blockade of viral core particle maturation [63].

Assembly of the HIV-1 capsid depends on CA-CA interactions involving structural domains NTD and CTD (Fig. 20.11). The peptides CAI and CAC1 and derivatives, as well as small molecules such as α-hydroxyglycineamide (a C-amidated tripeptide), benzodiazepine-based compounds and CAP-1 have been identified as inhibitors of HIV-1 capsid assembly [29, 30, 65]. CAP-1 contains a urea-based scaffold and two substituted aromatic groups at the molecule ends, and acts in the late stage of the viral life cycle. When added to preformed HIV-1 particles, the drug has no inhibitory effect on HIV-1 infection. The CAI peptide was identified in a screen using a random peptide library. The CAC1 peptide was rationally designed based on structure and thermodynamic information to include most of the residues critically involved in the CTD-CTD dimerization interface that participates in mature HIV-1 capsid assembly [65]. CAP-1, CAI, CAC1 and their combinations and/or derivatives were able to partially inhibit HIV-1 infectivity in cultured cells.

One of the limitations of CAI and other peptide inhibitors of capsid assembly is their inability to penetrate cells. Chemical modifications consisting of covalent crosslinks between amino acids found in those inhibitors led to the development of conformationally restricted peptides that showed increased affinity for monomeric CTD and improved delivery to target cells. Examples of those "stapled" peptides are NYAD-1 (derivative of CAI) and NYAD-201 [65]. NYAD-1 inhibits the formation of both immature and mature virions. At low micromolar concentrations, this drug interferes with early events in the viral life cycle. Despite their higher solubility and low toxicity, further development of HIV-1 CA-derived natural or stapled peptides is limited by their relatively modest binding affinities [30, 66]. Also, problems related with the general metabolic instability of peptides may need to be addressed by using peptidomimetic compounds.

A high-throughput antiviral screening campaign led to the identification of a cluster of structurally related compounds that inhibited HIV-1 replication at submicromolar concentrations by targeting a post-entry stage prior to reverse transcription. Among them, PF-3450074 (or PF74) triggers premature HIV-1 uncoating in target cells [67]. Interestingly, point mutations in CA that stabilize the HIV-1 core conferred strong resistance without influencing inhibitor binding. Structural studies of the complex of the CA N-terminal domain and PF74 (PDB entry 2XDE) revealed a new binding site, different from those described for CAP-1 (PDB entry 2PXR) or CAI (PDB entry 2BUO) (Fig. 20.11), and several *in silico* screened inhibitors [68]. PF74 increased the rate of HIV-1 CA multimerization *in vitro,* while CAI and CAP-1 decreased this rate under the same assay conditions. In a model of an assembled CA hexamer in complex with PF74, an indole group

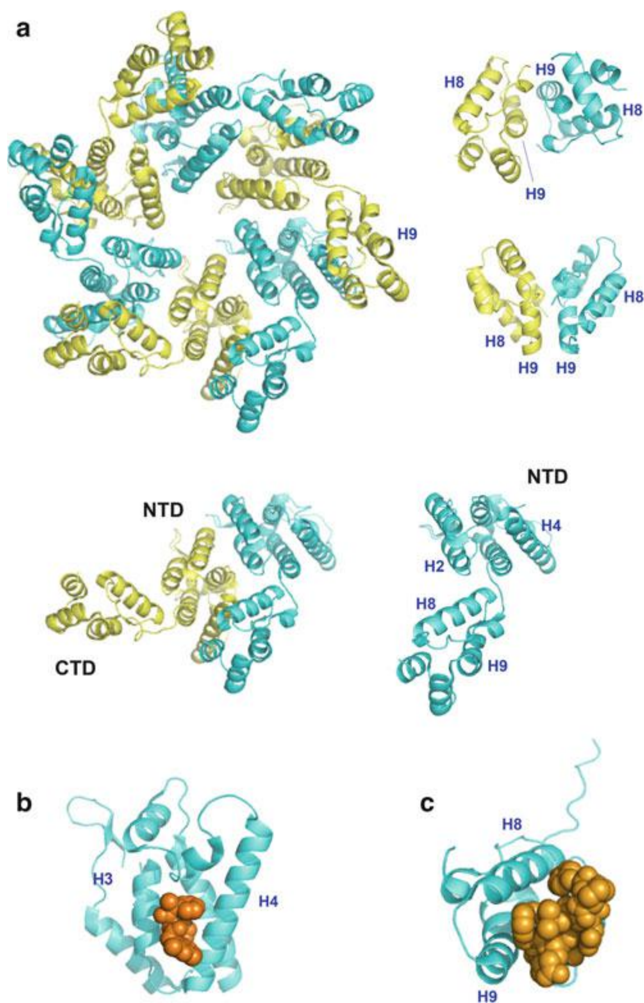

Fig. 20.11 HIV-1 capsid structure as a target of specific inhibitors. (**a**) Structural model of the HIV-1 CA hexamer [64] (coordinates taken from PDB entry 3GV2). The cartoons below show the structures of a CA dimer and a monomer, indicating the location of the structural domains NTD and CTD. Helices H2, H4 and H8 contribute to the NTD-CTD and NTD-NTD interfaces. CTD-CTD interactions are important for the formation of the capsid lattice by connecting hexameric rings. CTD dimerization is largely dependent on interactions between H9 helices (CTD dimers shown on the right are models obtained with data from the PDB entry 1A43). (**b**) Structure of a monomeric CA NTD bound to PF74 (PDB entry 2XDE). (**c**) Structure of a monomeric CA CTD bound to CAI (PDB entry 2BUO). All figures were produced using PyMol (http://www.pymol.org/)

protruding from the N-terminal domain was found to localize to the interface between capsid monomers suggesting both early and late stage effects on viral propagation. The N-terminal domain of CA is also the target of novel benzodiazepines that prevent virus release or benzimidazoles that inhibit the

formation of the mature capsid [69]. These compounds bind to a different site as compared with PF74 and have no effects at the early steps of viral replication. Benzimidazol inhibitors are expected to interfere with the formation of CA_{NTD}/ CA_{CTD} interfaces in the CA hexamer [69].

20.7 Perspectives and Conclusions

Despite the recent success in developing new antiviral drugs, particularly against HIV and HCV, there are still important challenges ahead. Drug resistance constitutes a frequent obstacle that limits the efficacy of antiviral treatments. As learnt from the HIV experience, monotherapies should be considered suboptimal therapies and combination therapies are expected to prevail in the future treatment of viral infections. Good combination therapies rely on the exploitation of multiple targets and/or mechanisms of action. A better knowledge of the biological processes involved in specific viral infections, including interaction with specific host factors, is required to develop efficient screening methods and to identify viable targets. A wealth of information derived from massive sequencing techniques applied to humans and pathogens will be probably helpful in this process. Drug efficacy, toxicity and cost are also issues that need to be considered in the development of future antiviral drugs.

From a technical point of view, high-throughput X-ray crystallography and structural analysis, computational modeling and better synthetic methods will provide tools to accelerate drug discovery, once the target mechanism has been validated. The rapidly increasing availability of high-resolution structures of viral particles, and substantial advances in our understanding of their morphogenesis and conformational dynamics are opening new avenues for the design of novel antiviral agents. Although the odds of finding effective inhibitors of novel targets have probably increased, the outcome still remains unpredictable.

Although HIV and HCV will probably continue to be major actors in the drug development field, other viral pathogens such as influenza virus and flaviviruses (*e.g.* dengue virus) will probably gain attention in the coming years due to their general impact on human health.

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Also especially recommended for further reading are references [1, 3, 18, 35] *listed above*.

Chapter 21
Design of Novel Vaccines Based on Virus-Like Particles or Chimeric Virions

Juan Bárcena and Esther Blanco

Abstract Virus-like particles (VLPs) are formed by viral structural proteins that, when overexpressed, spontaneously self-assemble into particles that are antigenically indistinguishable from infectious virus or subviral particles. VLPs are appealing as vaccine candidates because their inherent properties (*i.e.*, virus-sized, multimeric antigens, highly organised and repetitive structure, not infectious) are suitable for the induction of safe and efficient humoral and cellular immune responses. VLP-based vaccines have already been licensed for human and veterinary use, and many more vaccine candidates are currently in late stages of evaluation. Moreover, the development of VLPs as platforms for foreign antigen display has further broadened their potential applicability both as prophylactic and therapeutic vaccines. This chapter provides an overview on the design and use of VLPs for the development of new generation vaccines.

Keywords Virus • Virus-like particles • VLPs • Chimeric VLPs • Viral nanoparticles • Capsid structure • Structural proteins • Vaccines • Immune response • Multimeric presentation • Antigen display • Epitopes • B-cell • T-cell

Abbreviations

ANTXR2	Anthrax toxin receptor 2
APC	Antigen-presenting cell
BTV	Bluetongue virus
CpG-ODN	CpG-containing oligodeoxynucleotide
CPMV	Cowpea mosaic virus
CPV	Canine parvovirus

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cryo-EM	Cryo-electron microscopy
CTL	Cytotoxic T cell
DC	Dendritic cell
FHV	Flock house virus
GFP	Green fluorescent protein
HBcAg	Hepatitis B core antigen
HBsAg	Hepatitis B small surface antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HRV-14	Human rhinovirus 14
IBDV	Infectious bursal disease virus
MEV	Mink enteritis virus
MHC	Major histocompatibility complex
MIR	Major immunodominant region
PA	Protective antigen
PAMP	Pathogen-associated molecular pattern
PCV2	Porcine circovirus type 2
PRR	Pattern-recognition receptor
TLR	Toll-like receptor
VLP	Virus-like particle

21.1 Introduction

Vaccination is one of the most important and cost-effective methods of preventing infectious diseases. To date, no other method in human or veterinary medicine has had such an impact in reducing morbidity and mortality and increasing the overall well-being of humans and animals [1–5]. Life-threatening and devastating diseases, such as smallpox, have been eradicated by vaccination campaigns and the incidence of many others has been greatly reduced. Historically, this notable achievement has been brought about by an essentially empirical approach. Most of currently licensed vaccines are still produced by relatively simple methods of reducing the pathogen's virulence. These include biological attenuation of virulence, for example by repeated passage of viruses in cell-culture or embryonated eggs (live-attenuated vaccines), and chemical inactivation of whole microorganisms (inactivated or "killed" vaccines). Upon administration, these classical vaccines, especially the live-attenuated ones, frequently induce a spectrum of immune responses which closely resembles that elicited by the actual infectious agent: a potent B-cell mediated (or humoral) immune response plus a T-cell mediated (or cellular) immune response (Fig. 21.1), eventually conferring life-long protection of the vaccinated host against subsequent infection by the corresponding pathogen.

Nevertheless, in spite the successful development of numerous efficient classical vaccines their use presents relevant drawbacks regarding safety and other issues. There is a limited but present risk of reversion to a virulent phenotype *in vivo*, incomplete inactivation, or accidental pathogen release from vaccine manufacturing facilities, any of which can lead to spreading of the disease the vaccine was intended to prevent. Examples of this have been observed involving classical vaccines against poliovirus or foot-and- mouth disease virus [6, 7]. Live-attenuated vaccines are contraindicated for use in immunocompromised individuals or newborns. Besides, in the veterinary field, the use of classical vaccines do not usually allow for a clear-cut serological discrimination between vaccinated and pathogen-infected animals, a mandatory requirement to gain access to the international market of livestock products. These concerns have led many countries to a non-vaccination policy against important livestock diseases, that relies on slaughtering infected and contact herds, together with strict limitations on animal movements. Consequently, livestock populations are highly susceptible to such diseases, and there is a serious risk of reintroduction, as has happened repeatedly during the last years in several European countries, with diseases such as classical swine fever or foot-and-mouth disease. Another critical issue regarding the production of both human and animal classic viral vaccines is the need to develop a cell culture system susceptible of infection by the viral pathogen, that has not been always possible and/or advisable due, for example, to technical or safety reasons. Finally, the traditional strategies have shown their limits for the development of vaccines against organisms with complex pathogen-host interactions, that profit from undermining, evading, or misdirecting the host's immune response, such as human immunodeficiency virus (HIV). Hence, the focus of modern vaccinology is the development of alternative vaccine approaches circumventing the limitations of classical vaccines.

The advent of genetic engineering techniques and new biotechnological methods have enabled the development of synthetic or subunit vaccines, based on the use of isolated components of pathogens, such as recombinant immunogenic proteins or synthetic peptides, often portions of virus capsids. Subunit vaccines are considered an intrinsically safe approach as they are based on non-replicating immunogens and are, thus absolutely non-infectious. Importantly for veterinary use, they allow for discrimination between vaccinated and pathogen-infected animals (the so called marker vaccines). However, safety comes at a price. Vaccines based on isolated, soluble components of viruses and other pathogens are usually poorly immunogenic *per se*, requiring the administration of frequent and large doses of antigen. Moreover, they usually need to be formulated with potent immune-stimulating adjuvants, which may cause considerable side effects such as toxicity or pain.

Basic studies on virus structure and assembly (see Chaps. 2, 10 and 11) led to the experimental observation that many viral structural proteins have the intrinsic ability to self-assemble into virus-like particles (VLPs). These VLPs have not only been used in fundamental studies on capsid assembly, conformational stability and dynamics, and disassembly, but have also led to better immunological mimics of whole-virus particles compared to capsid subunits, resulting in improved effectiveness as vaccines, and leading to a renaissance in vaccine development. VLP

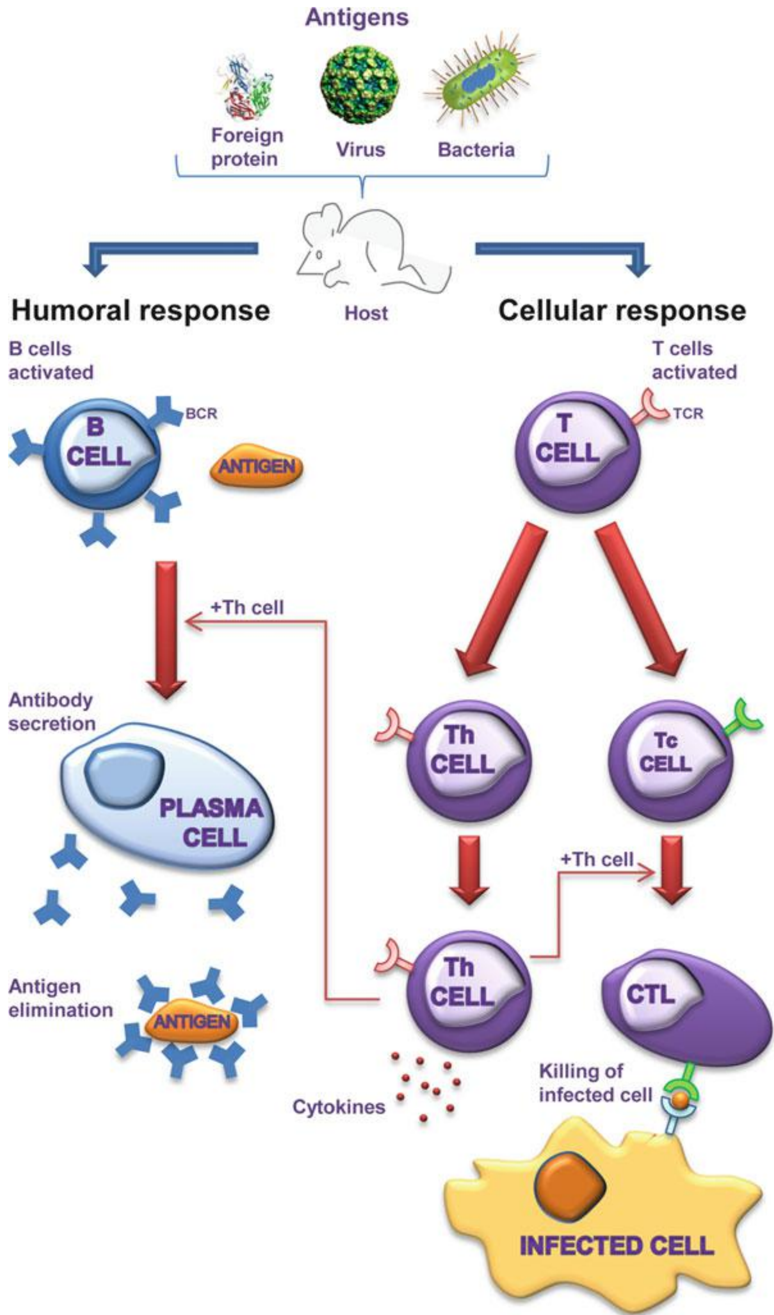

Fig. 21.1 The humoral and cell-mediated branches of the immune response. The humoral branch comprises lymphocytes of the B-cell lineage. Antibodies are the effector molecules produced by this response. The process begins with the interaction of B-cell receptors (*BCRs*) with antigens. Binding of the antigen promotes differentiation into antibody-secreting cells (plasma cells). The cell-mediated branch comprises lymphocytes of the T-cell lineage. T-helper cells (*Th cells*) and

based-vaccines combine many of the advantages of whole-virus-based and recombinant subunit vaccines, exhibiting a high safety profile. VLPs, produced using recombinant protein expression systems (thus avoiding the need to handle large amounts of pathogenic microorganisms), can stimulate strong B and T-cell immune responses and have been shown to exhibit self-adjuvanting abilities. These characteristics have made VLPs attractive stand-alone vaccine candidates for many viral diseases [8–13]. In addition, VLPs can also be used as platforms for the multimeric display of foreign antigens of interest, derived from viruses or other pathogens. This strategy couples delivery of the chosen antigen with the ability of VLPs to induce strong immune responses, thus acting as efficient adjuvants [14–17].

Until recently, vaccines were exclusively used to prevent infectious diseases (prophylactic vaccines). This situation is evolving rapidly and many efforts are currently focused on developing therapeutic vaccines to target diseases such as cancer, allergy and inflammatory autoimmunity, or even new indications like vaccines against nicotine dependence or hypertension [18–20]. In recent years, mechanisms underlying the outstanding immunogenicity of viruses have been elucidated, identifying important key parameters, like their size, particulate nature, their highly repetitive and ordered structures (see Chap. 2), or their ability to activate critical subsets of cells from the innate immune response, which in turn triggers appropriate conditioning of the adaptive immune response [21–23]. These features now serve as a paradigm for rational, virus structure-based vaccine design, and many new-generation tailor-made vaccines based on VLPs are attempting to harness these viral properties to target a wide array of human an animal diseases [23, 24]. This chapter provides an overview on the development and use of VLPs as vaccines for prophylactic and therapeutic applications.

21.2 Immunology of Vaccines, an Overview

What makes VLPs good vaccine candidates? In order to answer this question it is important to briefly review how vaccines induce a protective immune response.

The immune system has evolved to distinguish between noninfectious self and infectious nonself. Accordingly, it can mount strong immune responses to microbial infections but usually fails to respond to self molecules. Unravelling the mechanisms used by the immune system to discriminate self from nonself is critical for our understanding of diseases and our ability to rationally design vaccines.

The immune system is composed of the innate (nonspecific) and adaptive (specific) branches [21, 25]. The innate immune system represents the first line of

Fig. 21.1 (continued) cytotoxic T cells (*CTLs*) are the key effectors of this response. The process begins when T lymphocytes recognise antigens on the surface of antigen presenting cells (*APCs*). The Th cells recognise antigens bound to MHC class II molecules and produce cytokines that promote activation of other lymphocytes (both B and T cells). Once activated by Th cells, Tc cells differentiate into CTLs that can kill pathogen-infected cells

host defense against microbial infections. After a pathogen breaches the host's physical barriers such as mucosal surfaces or skin, the immune system mounts an early, innate immune response within minutes. Cells of the innate immune system, like dendritic cells (DCs) and macrophages, known as antigen-presenting cells (APCs), can recognise general characteristics of the pathogen and initiate a response. Pathogen-associated molecular patterns (PAMPs) are molecules or motifs that are common to many pathogens and generally not present in the host, such as lipopolysaccharide, which can be found on the cell wall of many bacterial species, double-stranded RNA or unmethylated CpG motifs, which are normally associated with virus infection. PAMPs can be recognised by Toll-like receptors (TLRs) and other pattern-recognition receptors (PRRs) which are present in host cells. The multivalent display and highly ordered structure exhibited by many pathogens, notably icosahedral virus surfaces that are essentially two-dimensional crystals, are also recognised as PAMPs, resulting in increased immunogenicity. PAMPs trigger innate immune sensing mechanisms and stimulate antigen uptake and processing by APCs. Subsequently, activated APCs present the antigens to cells of the adaptive immune response.

Adaptive immunity represents the second line of immunological defense and takes longer to develop, on the order of days. Once the antigen is delivered to the adaptive immune system and stimulates the proliferation (clonal selection and expansion) of antigen-specific effector cells, these cells begin eliminating the pathogen and infected cells. The adaptive response is also the basis for immunological memory, which is important for ensuring a fast and strong response when an infectious pathogen is encountered again (secondary infections). Vaccines use this process by introducing the immune system to a specific pathogen, stimulating the formation of specific memory cells and antibodies, which are then available to rapidly recognise the natural pathogen upon future exposures, resulting in its elimination and termination of the disease. The primary players of the adaptive immune response are B and T cells (lymphocytes) (Fig. 21.1). The key function of B cells is the production of antibodies. The role of T cells is more diverse ranging from lysis of infected cells, carried out by the cytotoxic T cells (CTLs), to secretion of cytokines, stimulation of B cells and other regulatory functions, performed by helper T cells (Th cells). The contribution of B cells *versus* T cells, and CTLs *versus* Th cells to resolution of infection varies from pathogen to pathogen and from primary to secondary responses [25]. Although it is difficult to generalise, antibodies are usually more important during secondary infections. In contrast, T cells are more important during many primary infections and, in particular, for the containment of chronic infections. Thus, for prophylactic vaccines, which aim at inducing effector mechanisms that protect from reinfection, the induction of neutralising antibodies is usually the most important factor. In contrast, for therapeutic vaccination against chronic infections, the emphasis is usually the stimulation of CTL responses. Hence, rational design of vaccines entails knowledge on the type of immune response to be evoked, and this in turn has structural implications for the design of new generation vaccines.

21.3 The Role of Size, Geometry and Molecular Patterns in Vaccine Design

The key properties of viruses that are responsible for eliciting potent immune responses and may be used as framework for vaccine design include their size, geometry, and the ability to induce innate immunity with appropriate conditioning of the adaptive immune responses [26].

The dimensions of vaccine antigens vary greatly [27–29]. The smallest (<10 nm) are protein or subunit antigen vaccines. Often, such antigens are formulated with adjuvants (such as alum and Freund's adjuvants) to form larger particles or aggregates. Supramolecular particulate antigens, such as VLPs and nanoparticles, are larger (20–200 nm). Small liposomes, such as virosomes (VLPs derived from viruses with lipid envelopes) are approximately 100–200 nm. Antigens presented in the context of microparticles, liposomes, water in oil emulsions, mineral salts and whole-cell vaccines are the largest (100 nm–20 μm).

The size of antigens is an important factor for their efficient uptake by APCs [26–29]. Particulate antigens, such as whole-cell vaccines, virosomes and VLPs, or antigens formulated in particulate adjuvants, such as liposomes and microparticles, have large surfaces with charge, hydrophobic or receptor-interacting properties, enabling better interaction with APCs. Thus, in contrast to small soluble protein antigens, pathogen sized particles and protein aggregates may efficiently be taken up by APCs. Following uptake, antigens are processed in the endosomal-lysosomal compartments of APCs, where they are degraded into peptides. These peptides are then loaded onto major histocompatibility complex (MHC) class II molecules and are subsequently transported to the cell surface for stimulation of Th cells (Fig. 21.2). Additionally, through the process termed cross-presentation, DCs (a special subset of APCs) have the ability to process and present the vaccine antigen *via* MHC class I molecules, to induce the priming of CTL responses. Soluble protein antigens are usually only inefficiently cross-presented, but particulate antigens that are similar in size to, or larger than, viruses efficiently reach the MHC class I pathway without intracellular replication, and their peptides are efficiently cross-presented (3–4 orders of magnitude higher than peptides from soluble antigens). Processing of particulate antigens that are 20 nm–3 μm in diameter has been documented and there is no clear indication that there are preferred sizes within this range. It is therefore possible that APCs have evolved to effectively process any antigen with dimensions that are similar to pathogens, ranging from viruses (20–100 nm) to bacteria and even cells (in the micrometer range). So, generating particulate vaccines may enhance the uptake, processing and presentation of the antigens by APCs.

Vaccines are usually injected subcutaneously or intramuscularly. However, adaptive immune responses are mainly induced in the secondary lymphoid organs. So, the transport of antigens through the lymphatic system from the peripheral tissues to lymphoid organs is important for vaccine design. Molecules of $20-200$ nm efficiently enter the lymphatic system through diffusion and drainage, with an optimal size being

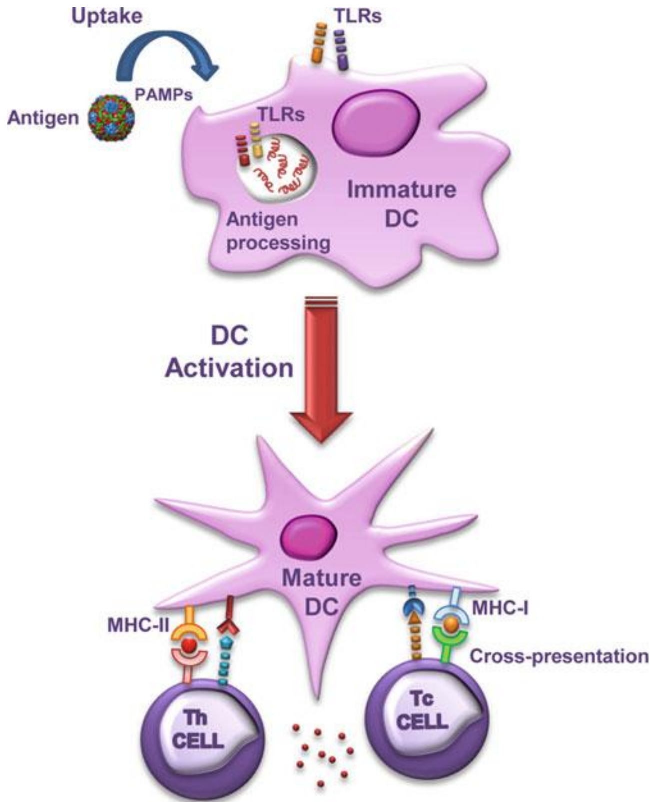

Fig. 21.2 Innate and adaptive immune systems are intimately connected. PAMPs present at the surface of pathogens stimulate antigen uptake and processing by special subsets of APCs, DCs, resulting in upregulation of DC maturation markers. Mature DCs present pathogen-derived antigens to naive Th and Tc cells *via* MHC class-II and class-I molecules, respectively. Secretion of cytokines by DCs stimulates differentiation into B and T effector cells resulting in antibody release and CTL responses

~40 nm, just within the size range of viruses and VLPs. By contrast, particles that are larger than 200 – 500 nm do not efficiently enter lymph capillaries in a free form. Instead, large particles need to be carried into the lymphatic system by specialised cells (particular subsets of APCs), which can squeeze through openings between overlapping endothelial cells. Therefore, size determines whether particles freely drain to lymphoid organs or arrive at these sites in association with cells, and this has major implications for vaccine design and immune cell targeting. As antigens with virus-like dimensions (and smaller) most efficiently reach the lymph nodes in a cell-free state, vaccine delivery systems with similar dimensions will facilitate direct interaction of antigens, in an authentic native configuration, with follicular B cells. This property dramatically increases their ability to induce antibody responses, and it is crucial for the induction of antibodies recognizing conformation-dependent epitopes. In addition, for the induction

of CTL responses, specific subsets of DCs ($CD8^+$ lymphoid DCs) that are essentially restricted to lymphoid organs, must be loaded with antigen. Hence, again particulate antigens with virus-like dimensions are preferable, since antigen size limits the ability of antigens to directly reach the adequate lymph node compartments.

Regarding the structure of antigens, most viral surfaces, as well as bacterial components such as the flagellum, consist of one or a few proteins and consequently are repetitive and highly organised in nature. Both the innate and adaptive immune systems recognise such highly repetitive structures as PAMPs. B cells, for example, recognise specifically and respond strongly to the ordered structure characteristic of viruses [30]. Most importantly, highly repetitive surface patterns efficiently cross-link B cell receptors on the surface of B cells, which represents a strong activation signal for these cells, leading to a strong antibody response [31]. It has been shown that 15–20 immunogenic molecules (haptenated polymer molecules) that are spaced 5–10 nm apart, is an ideal geometry for optimal B cell activation and this is similar to the average spacing of viral coat proteins in virion surfaces [32]. This also holds true for peptides that are conjugated to VLPs, where similar numbers and distances apply [31, 32]. Moreover, the stimulation of B cells by repetitive, highly organised antigens can be strong enough to elicit Th cell-independent induction of antibody responses (T-cell independent B-cell antigens). Therefore, these antigens can activate B cells more efficiently and at much lower concentrations than monomeric antigens.

Finally, it has become apparent that the innate and adaptive immune systems are intimately connected (Fig. 21.2). No relevant adaptive immune response will be mounted unless certain types of APCs, like DCs, are activated, resulting in a rapid release of inflammatory cytokines, and up-regulation of costimulatory molecules, which all serve to condition and shape the subsequent adaptive response [33]. For instance, cross-presentation alone is not sufficient to drive induction of a CTL response, as DCs need to be previously activated (indeed, non-activated DCs presenting antigen to T cells induce T cell tolerance rather than immunity). In this regard, several viruses and VLPs have been shown to induce the phenotypic and functional maturation (activation) of DCs [34], by triggering TLRs which recognise viral-specific PAMPs (Fig. 21.2). Interestingly, human papillomavirus (HPV) derived-VLPs, but not unassembled recombinant L1 protein (the constituent monomer of HPV VLPs), can activate DCs directly [35], suggesting that the size and organised structure of VLPs are important parameters to induce DC activation. However, it should be noted that different VLPs diverge in their ability to induce maturation of DCs, even if the VLPs are similar in size and structure (*i.e.*, VLPs from different polyomavirus species) [36], indicating that other factors that are VLP-specific can also affect the outcome of the interaction between VLPs and DCs.

Alternatively, an interesting approach currently being investigated is the use of PAMPs, in particular TLR ligands, as immune modulating adjuvants. These molecules have the ability to enhance B and T-cell responses induced by an antigen, offering advantages over existing conventional vaccine adjuvants. Virus-related PAMPs being developed as immune modulating adjuvants include CpG-containing oligodeoxynucleotides (CpG-ODNs), double stranded RNA derivatives and

analogues of poly(I:C). Unfortunately, the use of such TLR ligands as vaccine adjuvants is associated with several problematic issues. Because TLR ligands activate their cognate receptors which are present in many types of immune and non-immune cells, they have the potential to be toxic. Thus, co-administration of TLR ligands and antigen by simple mixing may lead to a degree of systemic exposure causing non-specific stimulation of the immune system, with resultant unwanted reactogenicity or immune related pathology. A strategy to solve these problems is to target the TLR ligands directly to DCs. This can be achieved by packaging viral TLR ligands within a particulate delivery system, providing an environment in which the ligand is stable and importantly, preventing its systemic distribution. Moreover, when combined with the antigen, the particulate nature of the delivery system serves to target both the antigen and the TLR ligand to the APCs, conveying them to the appropriate intracellular compartments, thus eliciting highly efficient activation of innate and adaptive immunity. VLPs, virosomes and liposomes have been used to co-deliver antigens with a variety of TLR ligands [22, 23, 31].

In summary, to induce potent immune responses, vaccines of viral size are preferable, as only particles in the nanometer range can reach lymph nodes and directly interact with B cells. Moreover, the antigenic epitopes should be displayed on the nanoparticles in an ordered and highly repetitive way to optimally activate B cells. Nanoparticle-sized vaccines are also preferable for the induction of T cell responses, as size limits the ability of particles to reach the appropriate DC subsets in lymphoid organs. Viruses (and some VLPs) have the ability to directly induce the activation of DCs by triggering TLRs. Delivery of TLR ligands by nanoparticles together with the antigen may facilitate activation of DCs, which is essential for the induction of T cell responses. Hence, vaccine carrier systems, such as VLPs, that mimic the size, geometry and PAMPs of viruses may be one possible way to optimally harness viral immunogenic properties, without the risks associated with viral infection (Fig. 21.3).

21.4 Virus-Like Particles (VLPs) as Antiviral Vaccines

21.4.1 Characteristics of VLPs

VLPs are supramolecular assemblies with a well-defined geometry, usually icosahedrons or rod-like structures, and diameters in the range of 20–120 nm [37] (Fig. 21.4). They are based on the natural intrinsic ability of many viral structural proteins to spontaneously assemble into multimeric structures when expressed using recombinant expression systems. VLPs mimic the organization and conformation of authentic native viruses (Fig. 21.5), with or without lipid envelope depending on the virus of origin and the viral structural protein expressed (*i.e.*, capsid proteins or viral envelope proteins), or in some cases subviral particles. Thus, VLPs are usually

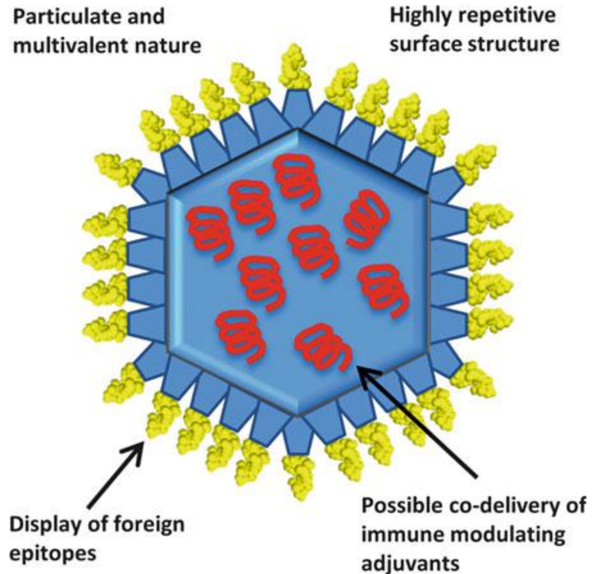

Fig. 21.3 Immunogenic features of VLPs presenting foreign antigens

structurally and antigenically indistinguishable from infectious virus particles, generally retaining the ability to bind and penetrate host cells, and are consequently highly immunogenic, but unable to replicate, since they lack the viral genome. In addition, immunization with VLP-based vaccines does not induce antibody responses to internal or non-structural viral proteins (which do not form part of the VLPs but are present in virus-infected cells, and therefore induce antibody responses in the infected host). This enables serological differentiation between vaccinated and virus-infected animals (marker vaccines), which represents an important feature for vaccines intended for veterinary use against notifiable diseases of livestock. Overall, these advantages have made VLPs attractive vaccine candidates against many viral diseases [8–13, 38–40].

The generation of VLPs derived from certain viruses has been strongly motivated by the lack of efficient cell culture propagation systems for those viruses, as was the case for hepatitis B virus (HBV), hepatitis C virus (HCV), HPV, or caliciviruses. An additional important reason for these efforts has been the possibility to substitute infectious viruses requiring high-level biosafety containment laboratories for their handling, like Ebola virus or Severe Acute Respiratory Syndrome virus, by VLPs. Hence, those VLPs have been used as efficient surrogates for virus diagnostics, basic research applications (like understanding the assembly or architecture of these viruses), and vaccine development. Moreover, an important challenge nowadays consists in the substitution of live vaccines, for example against influenza A virus, by an appropriate highly efficient and safe VLP-based vaccine.

In some instances, the VLPs are derived from internal structural viral proteins, like the HBV core antigen (HBcAg), or the Gag capsid proteins from retroviruses

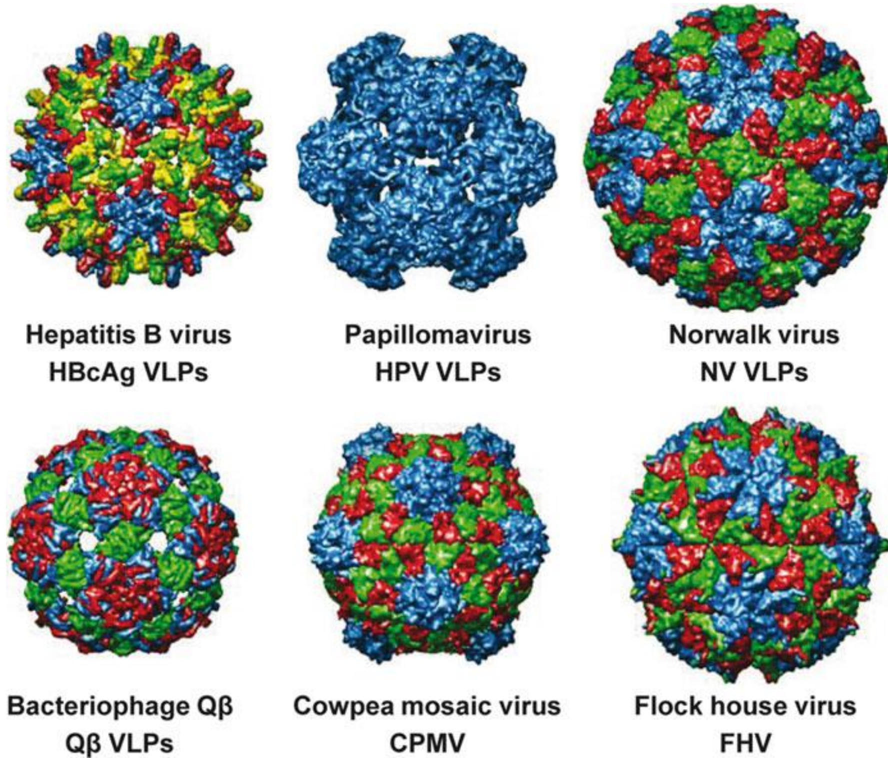

Fig. 21.4 Examples of viruses and VLPs mentioned in this chapter that have been developed as VLP-based vaccines or platforms for foreign antigen display. The images are coloured to distinguish the symmetry-related subunits and are taken from the VIPER database (http://viperdb.scripps.edu). Protein Data Bank (PDB) IDs: hepatitis B capsid, *1QGT*; Papillomavirus capsid, *1DZL*; Norwalk virus capsid, *1IHM*; bacteriophage Qβ, *1QBE*; cowpea mosaic virus, *1NY7*; flock house virus, 2Z2Q

(including HIV), which are capable of self-assembling into subviral particles. Such VLPs do not mimic the external surface structure of the corresponding native virions, and antibodies induced by them are not able to interfere with the course of virus infection. Hence, these VLPs are not suited *per se* for vaccine development against the viruses from which they were derived, but they can be used as scaffolds for foreign antigen presentation (see Sect. 21.5). Of the utmost interest for the development of antiviral vaccines are VLPs composed of viral surface proteins, usually corresponding to the external capsids of nonenveloped virions, particularly when virus-neutralising antibodies recognise those proteins and/or their assemblies. Examples of such VLPs are those derived from HPV, bluetongue virus (BTV), rotavirus, parvovirus, calicivirus, and infectious bursal disease virus (IBDV) [9, 13, 15, 17].

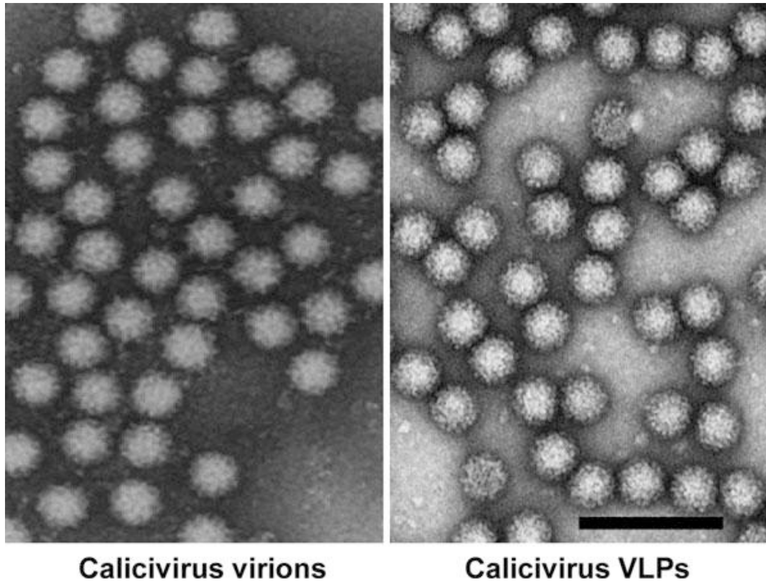

Fig. 21.5 Electron microscopy of negatively stained calicivirus particles: virions (*left*) and VLPs (*right*). Scale bar = 100 nm

21.4.2 Structural Classification of VLPs

VLPs have been produced for a wide range of taxonomically and structurally distinct viruses that infect humans and other animals, as well as plant and yeast viruses, and bacteriophages [13, 17, 41, 42]. These comprise viruses that have a single capsid protein, multiple capsid proteins, and those with or without lipid envelopes, indicating that the ability to develop VLPs does not appear to be limited to any type of virus family or by the complexity or structure of the virus particle. The largest number of VLPs have been obtained from viruses with single-stranded RNA genomes of positive polarity. Numerous families and genera of this most diverse structural class of viruses have been involved in the construction of differently sized and structured VLPs. Regarding organization and symmetry (see Chap. 2), this group of VLPs is represented by structures of two different symmetries: icosahedral (prevalent number of prototypes, 18 in total) and rod-like (three prototypes), derived from nonenveloped viruses, as well as by isometric VLPs without any distinct symmetry (three prototypes) derived from enveloped viruses [42].

Structural variants of VLPs are depicted in (Fig. 21.6). The simplest variant is represented by the self-assembly of a single viral structural protein into a single-shelled VLP. One of the most studied VLPs of structurally simple viruses is the HPV-derived VLP. HPV virions contain the major and minor capsid proteins (L1 and L2, respectively). The virus capsid (60 nm) contains 72 pentamers of L1

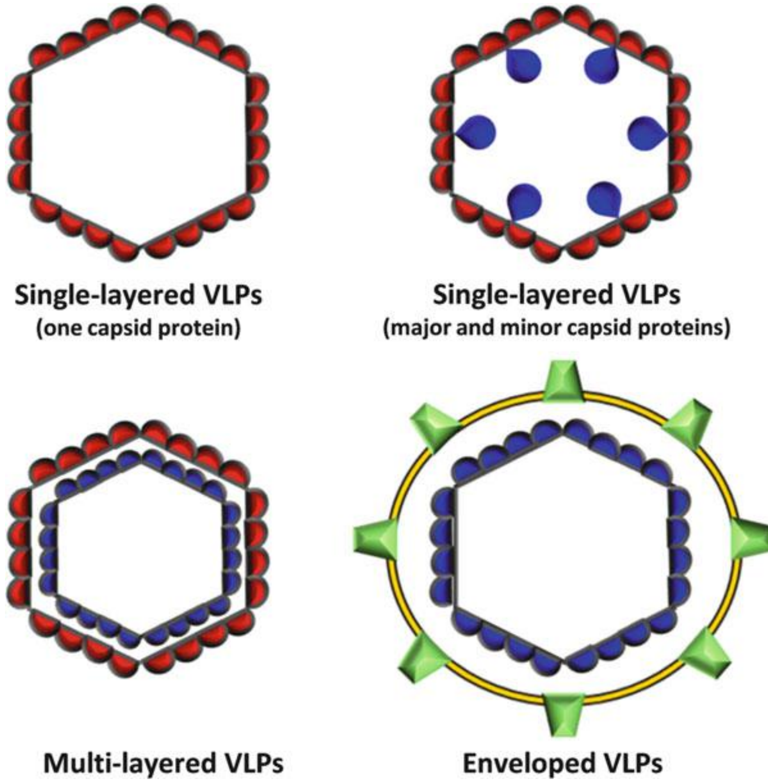

Fig. 21.6 Structural variants of VLPs

protein, centered on the vertices of a T = 7 icosahedral lattice (see Chap. 2), while L2 protein is present at about 1/30 of the abundance of L1. HPV VLPs are usually formed just by L1 protein, with morphological characteristics very similar to those of HPV virus capsids [43]. Parvovirus VLPs are also formed by a single protein (60 copies of VP2, the major viral structural protein). Other examples of VLPs formed by a single viral structural protein are those derived from the HBcAg, hepatitis E virus (HEV), calicivirus, nodavirus, circovirus, or bacteriophage Qβ [9, 17, 42]. Similarly, VLPs can be composed by both, a major and a minor protein forming a single shell, like in the case of HPV-derived VLPs composed by L1 and L2 proteins [44].

More complex multi-layered VLPs are generated by the co-expression of several viral structural proteins, like in the case of the BTV-derived VLPs (*Reoviridae* family). The icosahedral BTV virions are formed from non-equimolar amounts of seven structural proteins (VP1-VP7). However, only the four major structural proteins of the virus (VP2, VP3, VP5 and VP7) are necessary for the formation of double-layered VLPs, which are structurally indistinguishable from authentic virus particles [13]. VLPs derived from rotaviruses, also belonging to the *Reoviridae*

family, can be generated by co-expressing different subsets of viral structural proteins [11]. Expressing VP2 alone produces single-layered VLPs. Co-expression of proteins VP2 and VP6 that form the innermost and middle capsid layers of rotavirus particles, respectively, produces double-layered 2/6-VLPs. Co-expression of VP4 with VP2 and VP6 yields double-layered VLPs with VP4 spikes (2/4/6-VLPs). Co-expression of VP2, VP6, and VP7 produces triple-layered 2/6/7-VLPs, and finally, co-expression of VP2, VP4, VP6, and VP7 results in 2/4/6/7-VLPs that are complete triple-layered VLPs with the VP4 spike protein.

Another prototypic example of complex VLPs are enveloped VLPs containing unilamellar liposomes with embedded viral envelope proteins. The first VLP vaccine to be produced and characterised (in the early 1980s) consisted of isometric, spherical, pleomorphic particles with diameter between 17 and 25 nm, formed by the HBV small surface antigen (HBsAg), co-assembled with host cellular membranes [42]. Several other enveloped VLPs have been successfully developed by the simultaneous synthesis and co-assembly of two different viral protein species, *i.e*., capsid and envelope proteins. This is the approach used for the generation of VLPs derived from influenza virus, which contain both viral glycoproteins: hemagglutinin and neuraminidase, as well as matrix structural proteins. Other examples of enveloped VLPs are those derived from HCV, formed by the co-expression of core, E1 and E2 proteins, and retrovirus-based VLPs (like those of HIV), containing the Gag capsid protein and the envelope protein (gp120). The VLPs derived from viruses with lipid envelopes are sometimes referred to as virosomes [45].

21.4.3 Expression Systems for VLP Production

VLPs can be produced in many different production platforms, including mammalian cells, insect cells, plants, yeast, bacteria, and recombinant expression systems based on viral vectors: baculovirus, poxviruses, alphavirus replicons, etc. [41]. Currently, the most popular choice is expression in insect cells using the recombinant baculovirus technology [9, 46, 47]. This expression system has many advantages for VLP production. Large amounts of correctly folded recombinant proteins can be produced with mammalian-like post-translational modifications. Although yeast and bacteria cells can achieve similar yields, the complexity of the VLPs produced with the baculovirus expression system is remarkably higher (VLPs formed from the co-expression of up to five proteins). An additional advantage is that baculoviruses have a limited host range (namely for insects) and are hence safe for vertebrates. The design of recombinant baculoviruses is simple and fast, providing a high versatility to this expression system. This is important when producing vaccines for viruses whose surface proteins rapidly mutate, (*i.e*., influenza A virus), a fundamental requirement to contend with potential pandemics in a timely manner.

Although VLPs have been produced for a wide range of viruses, as indicated above, clearly not all are equally suitable for the development of vaccines. Even if a VLP-based product candidate has been shown to elicit a robust immune response under laboratory conditions, it might not be developed as a vaccine for widespread use, if its manufacturing process is not scalable or cost-effective. According to the experiences accumulated in the past years, some predictive considerations about the chances of success in adequate VLP production yields can be enumerated [41, 48]. Structurally simple non-enveloped icosahedral viruses are the most suitable candidates for successful high yield production of VLPs. VLPs made by the assembly of a single protein are usually able to be produced in large amount and high quality (*i.e*. HBcAg, parvovirus, calicivirus or papillomavirus, bacteriophage Qβ). An increase in the number of proteins to be expressed and assembled proportionally raises the difficulties of correct assembly [41], usually leading to lower yields of VLP production. The efficient assembly of capsids derived from proteins encoded by multiple discrete mRNAs (*i.e*., orbivirus, birnavirus), requires the co-expression of the interacting proteins in the vicinity of each other and within the same cell. On the other hand, capsid proteins that need to be processed from a polyprotein precursor in a timely fashion (monocystronic RNAs, *i.e*., picornavirus, flavivirus) are usually assembled with low yields, probably due to relatively inefficient processing of the precursors in recombinant expression systems and/or the toxicity of the viral protease required for processing. Finally, due to the inherent properties of the lipid envelope, production of enveloped VLPs is technically complex [41]. However, despite these considerations, progresses are being made, and it is expected that in the near future the integration of process optimization tools (*i.e*., molecular biology, genetic engineering and systems biology), will allow to overcome some of the current limitations affecting the large scale production of several types of VLPs [46, 47, 49, 50].

21.4.4 VLPs as Stand-Alone Vaccines

The most straightforward application of VLPs is to use them for vaccination against the virus from which they were derived. Currently, VLP-based vaccines against human diseases are in various stages of development, spanning from preclinical evaluation to market (Table 21.1) [9, 16, 17, 24].

VLP-based vaccines for HBV have been licensed commercially. Recombivax® (Merck) and Engerix® (GlaxoSmithKline), both licensed in 1986, are subvirion 22-nm spherical lipid-protein particles consisting of self-assemblages of the HBV small surface protein, HBsAg, co-assembled with membrane lipids, expressed in genetically engineered yeast (*Saccharomyces cerevisiae*). Although these vaccines were proved to be highly effective, they suffered from a lack of immunogenicity in a small percentage of patients (~5–10 % nonresponders), which was determined to be due to an absence of epitopes derived from PreS proteins (PreS1 and PreS2) on the surface of the VLPs. A more immunogenic VLP vaccine was subsequently

Table 21.1 Selected VLPs tested as vaccine candidates for different human and animal diseases

Virus	Family	Recombinant proteins	Envelope	Morphology of VLPs	Target species
Hepatitis B virus	*Hepadnaviridae*	Surface antigen	Yes	Isometric	Human
Human papillomavirus	*Papillomaviridae*	L1	No	Icosahedral, T = 7d, 1	Human
Influenza A	*Orthomyxoviridae*	HA, NA, M1, M2	Yes	Pleomorphic	Human
Norwalk virus	*Caliciviridae*	Capsid protein	No	Icosahedral, T = 3	Human
Hepatitis C virus	*Flaviviridae*	Core, E1, E2	Yes	Isometric	Human
Rotavirus	*Reoviridae*	VP2, VP4, VP6, VP7	No	Icosahedral, T = 13	Human
Rift valley fever virus	*Bunyaviridae*	N, Gn, Gc	Yes	Pleomorphic	Human
Porcine circovirus type 2	*Circoviridae*	Coat protein	No	Icosahedral, T = 1	Pig
Bluetongue virus	*Reoviridae*	VP2, VP3, VP5, VP7	No	Icosahedral, T = 13 l	Sheep
Rabbit hemorrhagic disease virus	*Caliciviridae*	Capsid protein	No	Icosahedral, T = 3	Rabbit
Porcine parvovirus, canine parvovirus, mink enteritis virus	*Parvoviridae*	VP2	No	Icosahedral, T = 1	Pig, dog, mink
Infectious bursal disease virus	*Birnaviridae*	VP2, VP3, VP4	No	Icosahedral, T = 13, 1	Chicken
Newcastle disease virus	*Paramyxoviridae*	NP, M, F, HN	Yes	Pleomorphic	Chicken
Avian influenza virus	*Orthomyxoviridae*	HA, NA, M1	Yes	Pleomorphic	Chicken
Nodavirus	*Nodaviridae*	Coat protein	No	Icosahedral, T = 3	Fish

developed that contained, in addition to HBsAg, the proteins PreS1 and PreS2, which are present in the native HBV envelope at lower levels than HBsAg. This new HBV VLP-based vaccine (Sci-B-Vac®, SciGene), expressed in the mammalian CHO cell line, was found to elicit a strong antibody response and 100 % seroconversion and seroprotection rates. Hence, a better mimic of the authentic virion surface structure resulted in broadened effectiveness of the HBV VLP-based vaccine [42].

The second type of commercially available VLP-based vaccines was developed against HPV, for the prevention of cervical cancer. These vaccines consist of 40 nm VLPs assembled from the HPV major capsid protein, L1. Gardasil® (Merck) is produced in yeast (*Saccharomyces cerevisiae*) and was licensed in 2006. Cervarix® (GlaxoSmithKline), is produced using the recombinant baculovirus expression

system and was licensed in 2009. Both contain mixtures of recombinant VLPs derived from different HPV types associated with genital infection (HPV types 6, 11, 16 and 18). The vaccines show great promise, as are almost completely protective against HPV types from which they are derived, and achieve partial protection against other phylogenetically related types. Since HPV types 16 and 18 are implicated in 70 % of cervical cancers, these vaccines are considered a breakthrough for preventing cervical cancer and are expected to drastically reduce the occurrence of this life-threatening disease in women [44].

Other VLP-based candidate vaccines for human diseases in an advanced stage of clinical development include those directed against human parvovirus (B19), Norwalk virus, rotavirus and influenza A virus [9, 16, 17, 24]. In the veterinary field there is currently one VLP-based vaccine commercially available against porcine circovirus type 2 (PCV2), Porcilis PCV® (Intervet), which was licensed in 2009. Other promising candidate vaccines in different stages of development include those against: BTV, IBDV, rabbit hemorrhagic disease virus, canine and porcine parvovirus, nodavirus, Rift valley fever virus, avian influenza virus or Newcastle disease virus [12, 38].

21.5 VLPs as Platforms for Foreign Antigen Display. Structure-Based Engineering of VLPs for Vaccine Development

As indicated in previous sections, VLPs can also be used as platforms for the multimeric display of foreign antigens, that can be incorporated into VLPs either by genetic fusion (chimeric VLPs) or by chemical conjugation (conjugated VLPs). In such cases VLPs serve both, as a presentation scaffold for antigens derived from other pathogens, in a suitable repetitive and highly organised configuration, and as adjuvants to boost the immune response. Ideally, the underlying immunogenic 'viral fingerprint' of the VLP is imparted to the attached antigen, making it as potent an immunogen as the VLP itself.

The poor immune response of many soluble antigens can be overcome by rendering them highly repetitive incorporating them to a carrier protein [51]. In contrast to monomeric and oligomeric carrier proteins commonly used for antigen presentation, such as bovine serum albumin and keyhole limpet hemocyanin, chimeric VLP structures are able to provide not only a high density of introduced foreign antigens per particle, but also a distinctive three-dimensional conformation, which is especially important for the presentation of conformational epitopes. Hence, the regular, repetitive pattern and correct conformation of inserted epitopes, are factors encouraging the development of VLPs as platforms for inducing immunological response against foreign antigens.

Insertion in specific sites of VLPs of continuous epitopes (those included in a short peptide segment) or continuous parts of discontinuous epitopes (those made up of several peptide segments in defined conformations) may alter the

conformational preferences of the peptide segment relative to the native ones (*i.e*., in their native protein or viral capsid), which may have dramatic effects on their immunogenicity as a part of the chimeric VLP. If complete discontinuous epitopes must be inserted in the VLP, the problem is severely compounded, as different peptide segments must be inserted in such a way that not only their conformational preferences, but also their geometric relationships are preserved in the chimeric VLP, relative to those in the native protein or viral particle. Detailed structural information and computational docking approaches may be critical for the rational design of highly immunogenic chimeric VLPs.

VLP chimeras have been extensively explored as vaccine candidates since mid-1980s [42, 52]. The interest in rational-based manipulations on chimeric VLPs was reinforced by the simultaneous development of recombinant DNA engineering techniques and structural knowledge about virions, capsids, and envelope proteins obtained by the use of high-resolution techniques (X-ray crystallography (see Chap. 4) and cryo-electron microscopy (cryo-EM) (see Chap. 3) [53]. Concurrently, research in immunology, molecular biology and structural studies led to the identification, characterisation and fine mapping of immunogenic epitopes derived from pathogens causing relevant diseases. X-ray crystallographic studies of antigen-antibody complexes as well as peptide-T-cell receptor interactions provided considerable insight into the structural basis of immunological recognition [54].

Currently a wealth of high-resolution viral structural information (see Chaps. 2, 3, 4, 5, 6 and 7) has facilitated the ability to modify VLPs deliberately so that they function essentially as molecular scaffolds for antigen presentation. VLPs derived from both double-and-single-stranded DNA and RNA viruses encompassing 14 different families of virus have been successfully used for the display of foreign antigens [14, 15, 17, 42] (Table 21.2). Results obtained using VLPs derived from different viruses, like HBV, HPV, parvovirus, calicivirus or bacteriophages have illustrated the potential of this approach to induce strong immune responses against foreign B and T-cell epitopes [14, 16, 17].

21.5.1 Chimeric VLPs Inducing Antibody Responses Against Target Molecules

In order to induce high-titer antibody responses effectively, target antigens must be displayed on the surface of VLPs, in immunodominant regions, at a high density. The genetic insertion of target sequences into viral structural proteins to generate chimeric particles is the most common method for displaying foreign epitopes on VLPs. Essentially, DNA fragments encoding continuous or discontinuous immunological epitopes are cloned into the genes encoding the self-assembly-competent polypeptides that form the VLPs. Upon assembly of the chimeric subunit proteins into supramolecular structures, the introduced epitopes are presented at a relatively high density (usually one copy per VLP subunit) and, ideally, in an accessible and

Table 21.2 Examples of VLPs and chimeric virions used as platforms for foreign antigen display

VLP platform or Viral nanoparticle	Foreign antigen	Incorporation method
HBcAg	Influenza A M2 extracellular domain, B- and T-cell epitopes of HCV, epitopes derived from malaria	Genetic fusion
HBsAg	Dengue virus envelope, HIV gp41epitopes,	Genetic fusion
Bovine papillomavirus	CTL epitopes of HPV and HIV, L2 HPV epitopes, Aβ peptide	Genetic fusion
HPV	HPV E7 oncoprotein	Genetic fusion
HPV	Aβ peptide	Chemical conjugation
Rabbit hemorrhagic disease virus	Model T-cell epitopes (*i.e*., Chicken ovoalbumin CTL epitope)	Genetic fusion and chemical conjugation
Porcine parvovirus	Model T-cell epitopes (*i.e*., CTL epitope derived from LCMV nucleoprotein)	Genetic fusion
Phage Qβ	Nicotine, angiotensin II, CCR5 extracellular domain	Chemical conjugation
CPMV	B-cell epitope (NIm-1A) of HRV-14, B-cell epitope of canine parvovirus	Chemical conjugation
FHV	ANTXR2 domain, ectodomain of G protein from Rift valley fever virus	Genetic fusion

conformationally relevant manner. An advantage of this chimeric VLP technique is that successful incorporation of a target epitope guarantees that the antigen will be displayed in the same conformation and at high density on the particle surface. This technique also has substantial advantages from a manufacturing standpoint. Chimeric particles can be purified using the same well-established methods used to purify unmodified parental VLPs.

The key to this technology is the identification of adequate permissive insertion sites to incorporate the target epitopes, within the primary sequence of the VLP subunits. That is, sites that do not compromise the correct folding and assembly of the VLP protein subunits, and do not alter the structural integrity and immunogenicity of the VLP. Obviously, depending on the type of foreign epitope to be displayed, the structural requirements of the insertion sites would be different. B-cell epitopes should be located at exposed sites on the surface of the particles, preferentially located at immunodominant regions, readily accessible for direct interaction with B-cells. However, T-cell epitopes do not need to be located at exposed sites within the VLP structure, because these epitopes have to be proteolytically processed by APCs before being presented to the target T-cells (as explained in Sect. 21.3), and thus can be inserted at nonaccessible permissive sites of the VLP. Hence, knowledge of the three-dimensional structure of the VLP scaffold is a critical requirement for successful generation of chimeric VLPs. If the three-dimensional structure of the VLP is not available, attempts can be made to locate suitable insertion sites in surface regions predicted on the basis of sequence analysis, generally at the N or C termini of the VLP protein subunits [55]. These sites are expected to raise fewer assembly problems for the resulting chimeric

VLPs. However, the immune responses obtained against the inserted foreign epitopes are often limited, most likely because the epitopes are not optimally exposed to the immune system at that locations.

Since many B-cell epitopes in their native environments are found at surface loops of the pathogen's proteins, this is often a good choice for the insertion of foreign antigens in VLP protein subunits. In some cases, proper folding and presentation can be accomplished by replacing exposed, immunodominant viral epitopes located at loop structures on VLPs, with the desired target epitope. However, generating chimeric VLPs is still largely empirical; it is almost impossible to predict whether individual peptides will be compatible with VLP assembly at defined insertion sites or whether the insertions will be immunogenic in the resulting chimeric VLP. For instance, it has been shown that minor displacements in the insertion site (two or three amino acid positions) can cause drastic changes in the immunogenicity of the inserted sequence [56]. Peptide insertions with high hydrophobicity, a high β-strand index, a large volume or a strong positive charge are prone to cause problems in VLP assembly. The success rate can be improved using combinatorial technologies. This type of approach was used to generate chimeric VLPs derived from the core protein of woodchuck hepadnavirus [57], enabling the identification of 17 different sites in the subunit protein that could be used to insert target sequences.

Another limitation attributed to the chimeric approach is that the size of the antigens that can be inserted into VLPs, in particular into their surface-exposed immunodominant regions, is usually restricted to short peptides. Chimeric protein subunits containing foreign peptide insertions longer than 20–30 amino acids often fail to assemble into VLPs. However, it should be noted that there are marked differences in the versatility of the different VLPs as antigen display platforms, regarding the size of the insertions they can tolerate. Relatively large insertions have been successfully incorporated into exposed sites in VLPs, like the 238 amino acids of the complete green fluorescent protein (GFP), that has been inserted in HBcAg-derived VLPs [42]. Other whole proteins or complete protein domains, with more relevance as immunogens to induce antibody responses, have been successfully inserted in chimeric VLPs, like a 120 amino acid-long polypeptide from the hantavirus nucleocapsid protein in HBcAg-derived VLPs, or a 395 amino acid portion of dengue virus envelope protein type 2, in HBsAg-derived VLPs [17]. Hence, in some cases, it is possible to display large antigens in the surface of chimeric VLPs. This might be important in order to increase the number of epitopes targeted by an individual chimeric VLP, or for targeting complex discontinuous B-cell epitopes.

The VLP derived from the HBcAg is the most extensively investigated chimeric carrier of foreign antigens to date [42, 52]. HBcAg polypeptide is 183–185 amino acids long (21-kDa). The organization of HBcAg derived particles is largely α-helical and thus, quite different from previously known viral capsid proteins with β-sheet jellyroll packings. The HBcAg monomer fold is stabilised by a hydrophobic core that is highly conserved among human HBV variants. Association of two amphipathic α-helical hairpins results in the formation of a dimer with a

four-helix bundle as the major central feature. The dimers are able to assemble into two types of particles, large and small, that are 34 and 30 nm in diameter and correspond to triangulation numbers T = 4 and T = 3, containing 240 and 180 monomers, respectively (see Chap. 2). The major immunodominant region (MIR) with the central amino acid positions 76–81 is located at the tips of the α-helical hairpins that form spikes on the capsid surface. In addition to the highly exposed MIR, the region between amino acids 127 and 133 is the next exposed and accessible epitope on the particle surface. This region is located at the end of the C-terminal α-helix and forms small protrusions on the surface of the HBcAg particle. Foreign antigens can be inserted at the N- and C-termini, and at the MIR (*i.e*., the tips of the VLP spikes), without affecting the ability of the resulting chimeric proteins to assemble into VLPs [42, 52]. The N-terminal end of HBcAg has been widely used as insertion site to display a variety of short foreign epitopes. The capacity for incorporating foreign peptides at this site is around 50 amino acids. The inserted epitopes are accessible to specific antibodies and in general terms, high levels of specific antibody responses are achieved. Regarding the C-terminal insertions, amino acid positions 144, 149, 156, 163 and 167 are the most frequently used sites for the incorporation of foreign epitopes. The capacity of the C-terminal end to accept foreign insertions usually exceeds 100 amino acids residues, depending on the structure of the inserted polypeptide. In some cases, quite large polypeptides have been successfully inserted at this location. A 559 amino acid long insertion, containing three copies of the HCV core protein, did not prevent self-assembly of chimeric proteins. However, the C-terminal end of HBcAg is not exposed at the surface of the VLPs and thus, chimeric constructions harbouring foreign antigens at this site usually induce weak to moderate specific antibody responses. Hence, this site is mainly used for the insertion of T-cell epitopes. The most interesting and promising site for foreign insertions is the MIR, which allows full exposure of the inserted peptides on the VLP surface. In addition, this insertion site has revealed an unexpected high capacity for the incorporation of large foreign polypeptides. The entire 120 amino acids long immunoprotective region of the hantavirus nucleocapsid protein, and the 238 amino acids long GFP (as indicated above), have been successfully incorporated into chimeric HBcAg VLPs at this insertion site.

Mosaic VLPs have been developed to aid in the assembly of complex VLP chimeras. These VLPs are typically formed by co-expression of a wild-type VLP subunit along with a chimeric VLP subunit. The resultant VLPs contain copies of both wild-type and chimeric subunits which are incorporated with differing proportions. The use of mosaic VLPs has enabled the formation of HBcAg VLPs containing fragments of up to 213 amino acids from the hantavirus nucleoprotein, or 163 amino acids from the HBV preS domain [42]. Alternatively, coexpression of VLP subunit proteins derived from different virus strains may be used to assemble mosaic VLP vaccines that generate antibodies that protect against multiple pathogen serotypes. This approach has been used for the generation of mosaic VLPs derived from HPV formed by the co-expression of L1 and L2 capsid proteins of both, HPV-6 and HPV-16 types. The resulting VLPs comprised all four co-assembled subunit proteins.

21.5.2 Chimeric VLPs Targeting Self-Antigens

Traditionally, antibody-inducing vaccines were used solely to provide prophylactic protection against pathogens. More recently, there has been a growing interest in the development of new vaccines with the goal of deliberately inducing antibody responses against self-molecules that are involved in chronic disease processes. These autoantibodies are expected to serve as competitive inhibitors to fight diseases. The ability to induce antibody responses against self-molecules is seemingly limited by the mechanisms of B-cell tolerance, which eliminate, desensitise or change the specificity of potentially self-reactive B cells, in order to prevent autoimmune disorders. However, B-cell tolerance is somehow inefficient; it has been estimated that approximately 20 % of long-lived mature B cells are self-reactive. Thus, antibodies against self-molecules can be induced by vaccination.

The main hurdle for eliciting a high titer antibody response against self-antigens is overcoming T-cell tolerance. Stringent T-cell tolerance mechanisms ensure that autoreactive B cells cannot receive T-cell help and, therefore, cannot proliferate and produce potentially damaging autoantibodies. Thus, one strategy for inducing antiself antibodies has been to immunise with self-antigens chemically conjugated or genetically linked to foreign T-helper epitopes. This approach requires large doses of antigen and the use of powerful adjuvants. In addition, antibody titers induced against the self-antigen are often very low and rapidly diminish, particularly in comparison with the responses induced by vaccines against foreign antigens. By contrast, self-antigens displayed on VLPs are inherently immunogenic at low doses and without exogenous adjuvants. It has been shown that self-antigens incorporated to VLPs can induce antibody titers up to 1,000-times higher than those induced by the same self-antigen linked to a foreign T-helper epitope. Moreover, VLP display can make a self-antigen as immunogenic as a foreign antigen presented in the same context. The magnitude of the antiself antibody responses correlates with the density at which the self-antigen is displayed on the VLP surface. In conclusion, B cell unresponsiveness can be overcome by antigens presented in a repetitive and highly organised 'foreign-like' manner. In fact, the immune system is largely unable to distinguish between self and foreign proteins based on antigenic epitopes, but does so based on antigenic structural organisation.

VLP display of self-antigens has been successfully used to target molecules that are involved in the pathogenesis of a variety of chronic diseases, including Alzheimer's disease, rheumatoid arthritis, hypertension, acquired immunodeficiency syndrome and certain cancers [18–20, 24]. Some of these vaccines have shown clinical efficacy in animal models and several are currently in clinical trials. However, a vaccine inducing an antibody response against a self-protein raises safety concerns that must be addressed.

Alzheimer's disease is a neurodegenerative disease that results in accumulation of plaques consisting of aggregated amyloid-β (Aβ) peptide, neurofibrillary tangles, and loss of neurons leading to dementia. One therapeutic strategy has been the use

of vaccination against Aβ to eliminate the accumulation of plaques in the brain in the hope of preserving neurons. The attachment of an Aβ peptide to papillomavirus VLP capsids induced high levels of specific antibodies and inhibited effective assembly of peptides into neurotoxic peptides *in vitro*. Aβ deposits were also reduced after immunization of a mouse model of Alzheimer's. However, clinical trials of another vaccine formulation against Aβ peptide resulted in development of encephalitis in a subset of patients, that was ascribed to T-cell responses associated with the vaccine. Hence, an effective Alzheimer's disease vaccine might be dependent on the consistent induction of high-titer anti-Aβ antibodies in the absence of inflammatory antiself T-cell responses. A VLP-based vaccine targeting a short peptide at the N-terminus of Aβ, which does not contain a T-cell epitope, could be one possible solution. Two different VLP-based Aβ vaccines targeting this epitope induced high levels of anti-Aβ antibodies, and were not associated with unwanted T-cell responses. Thus, the flexibility of the VLP display system allows the tailoring of vaccines, hopefully enabling to conform to both safety and efficacy requirements.

Another example is one potential therapeutic approach to prevent HIV infection, based on the production of autoantibodies against the HIV cellular receptor CCR5. It is an indirect route to vaccination, avoiding the problem of constant mutation of the viral antigens. A peptide from CCR5 was attached to HPV VLPs and when administered in mice, these particles initiated production of autoantibodies, which inhibited binding of the ligand and blocked infection of an indicator cell line expressing CCR5.

Vaccines directed against self-molecules hold promise for treating a variety of chronic diseases by the induction of antibodies targeting endogenous proteins. In contrast to passive vaccination with monoclonal antibodies, active immunisation with VLP-based vaccines displaying self-peptides induce long-lasting antibody responses, potentially providing affordable and convenient therapy for patients.

21.5.3 Chimeric VLPs Inducing Cell-Mediated Immunity

The use of chimeric VLPs as platforms to induce CTL responses against foreign antigens presents different requirements than vaccines aimed at inducing humoral responses. Unlike B-cell epitopes, T-cell epitopes do not need to be exposed on the surface of the particle or presented at high density, are included in single peptide segments (*i.e.*, they are continuous epitopes), and do not depend on their being stabilised in a specific folded conformation before being recognised by the corresponding immune system molecule. This enables diverse approaches for the generation of chimeric VLPs intended for this purpose. Chimeric VLPs can be generated not only by fusing target antigens to viral major capsid proteins (as were the examples cited in the previous sections), but also by making fusions

between target antigens and proteins that are minor structural components of VLPs, and are often not necessary for particle formation. In this case, the target antigens would not be displayed in high numbers per particle, and will usually be located facing the interior of the VLPs. This is not detrimental in the case of T-cell epitopes, and in fact, in some situations can be advantageous. Thus, targeting large polypeptides might be necessary in situations in which the most relevant target CTL epitopes in a protein have not yet been precisely mapped. Large peptides and even full-length proteins can be added to minor structural proteins without interfering with capsid assembly. For instance, the nonstructural HPV encoded oncoprotein, E7, was fused to the HPV minor capsid protein L2. When co-expressed alongside the HPV major capsid protein (L1), L2–E7 was incorporated efficiently into VLPs and because E7 was incorporated on the inside of the VLP, the fusion domain did not interfere with cell binding. Chimeric L1/L2–E7 VLPs induced strong CTL responses that protected mice from challenge with an E7-expressing tumor cell line [58].

As indicated in Sect. 21.3, in order to activate cytotoxic T-cells, DCs must also provide appropriate costimulatory signals that are only upregulated upon DC maturation. Certain VLP types, such as HPV VLPs, can provoke these signals by directly stimulating the phenotypic and functional maturation of DCs, and thus, are highly efficient inducing CTL responses. In addition, the immunogenicity of many VLPs can be improved by incorporating to the particles substances with adjuvant activity, like TLR ligands. Proof of concept was established using bacteriophage Qβ VLPs co-delivering TLR ligands and CTL epitopes to DCs in animal models [24]. It was shown that Qβ-VLPs devoid of any TLR ligands essentially failed to induce a CTL response, while Qβ-VLPs which were internally loaded with CpG-ODNs induced a robust response against the foreign CTL epitope incorporated to the VLPs (the p33 epitope from lymphocytic choriomeningitis virus glycoprotein).

In summary, numerous chimeric VLPs designed to present B and T-cell epitopes in this manner have been tested in preclinical research [9, 14, 16, 17, 19, 20, 24]. The final intended application of such vaccines spans human to veterinary use and prophylactic to therapeutic treatment. Epitopes have been derived from viral, bacterial, eukaryotic parasitic, and disease related self-molecules. Four of these chimeric vaccines have entered clinical trials: an anti-influenza A M2-HBcAg VLP vaccine, an anti-HIV p17/p24:Ty VLP (a chimeric VLP derived from the yeast retrotransposon Ty1), and two chimeric anti-malaria vaccines based on VLPs derived from HBcAg or HBsAg, displaying antigens from malaria proteins.

21.5.4 Chemical Conjugation of VLP-Based Assemblies

An alternative approach for displaying antigens on the surface of VLPs is the use of modular systems, in which the native VLP and target antigen are synthesised separately and then chemically conjugated *in vitro*, covalently or noncovalently, linking the antigen to the surface of preassembled VLPs (Fig. 21.7) (see also Chap. 22).

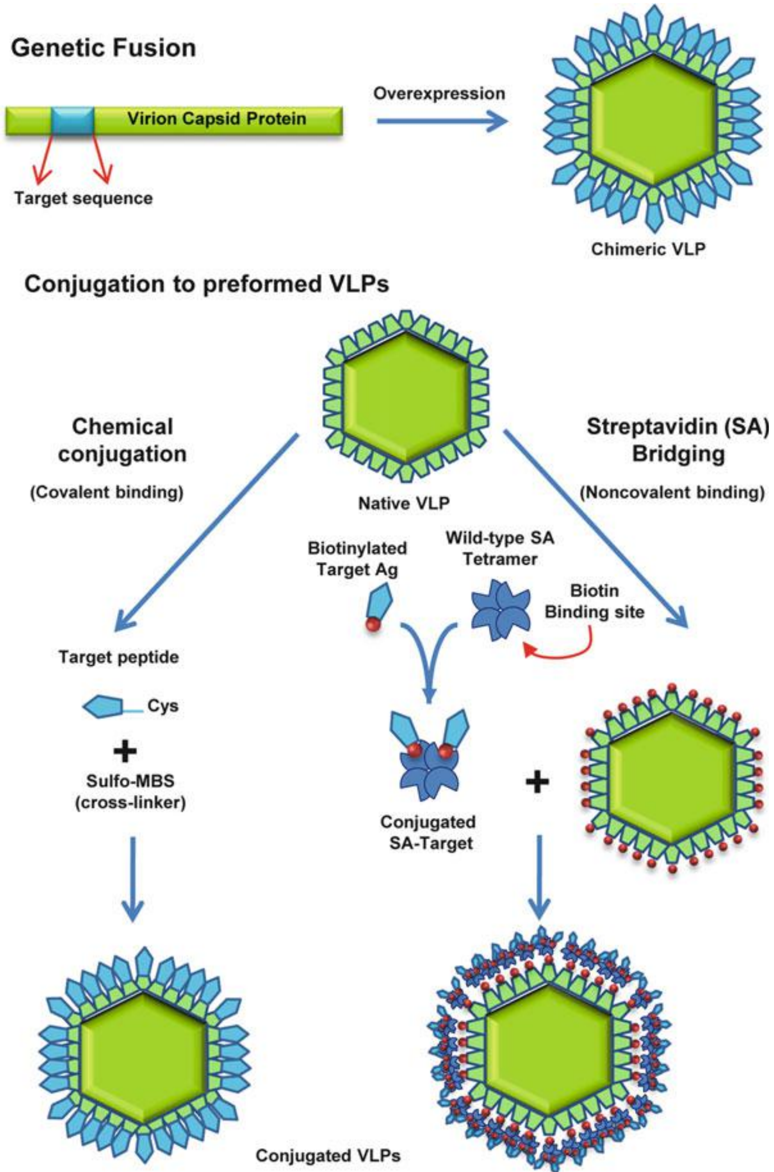

Fig. 21.7 Different approaches for displaying antigens on the surface of VLPs. For the genetic insertion of target sequences, the DNA encoding an antigenic peptide is cloned into the gene encoding the self-assembly-competent polypeptide that forms the VLPs. The resulting construction self-assembles into chimeric VLPs. Chemical conjugation techniques rely on the use of modular systems, in which the native VLP and target antigen are synthesised separately and then conjugated *in vitro*, covalently or noncovalently, linking the antigen to the surface of preassembled VLPs

An advantage of this approach is that the size and structure of the target antigen to be attached are not constrained by the requirements of the correct folding of the VLP monomers and particle assembly. Furthermore, chemical conjugation allows the attachment to the surface of VLPs of diverse kinds of target antigens: short linear peptides, cyclic peptides, full-length proteins, and even nonprotein targets, such as glycans or small haptens (organic molecules that can elicit an immune response only when attached to a protein carrier). A recent example is the antismoking candidate vaccine Nicotine-Qbeta (Cytos Biotechnology), which is produced by covalently coupling nicotine to the surface of VLPs derived from the bacteriophage Qβ. This vaccine is currently in late stages of clinical evaluation and it has been shown to induce high titer nicotine-specific antibody responses in immunised subjects [20, 24].

The conjugation techniques (see also Chap. 22) rely on the presence of addressable moieties on the surface of VLPs, typically amine or sulfhydryl residues. The most commonly used chemical techniques are those employed routinely for protein derivatization [59]: acylation of the amino groups of lysine side chains and the N -terminus, alkylation of the sulfhydryl group of cysteine, and, to a more limited extent, activation of carboxylic acid residues and coupling with added amines. Usually, surface-exposed lysine residues on virus capsid proteins are targeted for conjugation of foreign antigens. Covalent linkage can be achieved by the use of diverse chemical crosslinkers, normally, heterobifunctional conjugation reagents containing two distinct reactive groups that couple to different functional targets, one on the VLP and the other on the antigen. For example, using maleimidobenzoic acid sulfosuccinimidyl ester (Sulfo-MBS), cysteine-containing antigens can be conjugated at high density (*i.e.*, one to three molecules per VLP protein monomer) to exposed amines (lysine residues) on the surface of VLPs [60]. If needed, viruses and VLPs can be engineered to contain useful attachment sites on the surface of the particle [59, 61]. For instance, HBcAg VLPs, bacteriophage MS2 and cowpea mosaic virus have been mutated so that they contain a single surface-exposed reactive residue (lysine or cysteine) per capsid subunit, suitable for antigen conjugation [14, 59, 60]. To ensure that the antigen is coupled to the VLP in a directed and oriented fashion, peptide antigens can be engineered to contain either an amino- or carboxy-terminal amino acid linker sequence containing a free cysteine group.

For noncovalent conjugation of antigens to VLPs, exposed lysine residues can be biotinylated and then attached to biotinylated target antigens through the use of a streptavidin linking molecule [24]. Using this approach VLPs displaying peptides derived from self antigens (*i.e.*, Aβ, CCR-5 or TNF-α) have been obtained [24].

Hence, the flexibility of the chemical conjugation approach offers substantial inherent advantages. However, from the manufacturing point of view, it poses some drawbacks. Chemical linkage results in less than the 100 % of foreign antigen insertion achieved with the genetic fusion method, and is not as reproducible. The production process of conjugated VLPs therefore entails extra challenges and the quality control methods are inevitably more complex.

21.6 Use of Plant and Insect-Derived Chimeric Virions for Foreign Antigen Display

An interesting approach related to the VLP-based vaccine technology is the use of genetically engineered plant viruses as vectors for the presentation of foreign epitopes. In this approach the heterologous peptides are inserted by genetic fusion at exposed loops within viral capsid proteins, but using infectious viral clones for cell transfection, resulting in the formation of viable chimeric virus particles. In contrast with VLPs (in the strict sense frequently given to this definition), these chimeric whole-virus particles are replication-competent and are therefore infectious in plants, their natural hosts. The engineered viruses are propagated in plants and subsequently, the corresponding purified chimeric virions are used as immunogens in target animals. Since the plant viruses are unable to replicate in mammals, these chimeric virions behave similarly to VLPs in terms of safety and the immune response induced. Both, filamentous and icosahedral plant viruses have been successfully developed as foreign epitope presentation systems [62]. The most frequently used systems are cowpea mosaic virus (CPMV), tobacco mosaic virus, cucumber mosaic virus, alfalfa mosaic virus and papaya mosaic virus.

CPMV has been extensively studied for vaccine applications [63–65]. It is a comovirus in the picornavirus superfamily, with a capsid composed of two protein subunits termed small (S) and large (L). The S subunit is about 23 kD and folds into a jellyroll β-sandwich, while the L subunit folds into two jellyroll β-sandwiches, with a total mass of 41 kD. Sixty copies of both proteins form the virus capsid 30 nm particle with a $T = 3$ icosahedral symmetry. Knowledge of the detailed three-dimensional structure of the particle allowed the identification of suitable sites for the insertion of foreign peptides, and the availability of infectious cDNA clones enabled engineering the corresponding genetic changes in the viral genome. Thus, CPMV became the first plant virus to be successfully developed as a peptide presentation platform [65]. In most cases, the foreign sequence has been inserted into the most exposed loop of the virus surface, the βB-βC loop of the S protein, resulting in the display of 60 copies of inserted peptide on the surface of the particle. Other sites, such as the $\beta C'$-$\beta C''$ loop of the S protein and the βE-αB loop of the L protein, have also been used successfully. Provided the inserted peptide is less than 40 amino acids and has a pI below 9.0, the yields of modified particles are generally similar to those obtained with wild-type CPMV (up to 1 mg of particles per gram of infected leaf tissue).

CPMV particles have been genetically modified to display foreign epitopes derived from mink enteritis virus (MEV) and canine parvovirus (CPV) VP2 proteins, as well as HIV gp41 protein. These chimeric particles were shown to induce neutralising antibody responses when injected into experimental animals, and in the cases of the chimeric particles harbouring epitopes derived from MEV and CPV, the immune responses induced resulted in protection against viral challenges with the corresponding viruses. Likewise chimeric CPMV particles expressing a foreign antigen derived from the outer membrane protein F of *Pseudomonas aeruginosa*

were able to protect mice against challenge by two different immunotypes of *P. aeruginosa* in a model of chronic pulmonary infection.

Biochemical analysis of chimeric CPMV virions with foreign antigens inserted at the βB-βC loop or the βC′-βC″ loop identified a proteolytic cleavage event near the carboxy-terminus of the inserted sequence, which appears to be position, rather than sequence, dependent. This cleavage does not result in the loss of the epitope from the surface of the virion, but as a result the epitope is only anchored to the viral surface by its N-terminus.

An interesting feature of CPMV as an antigen presentation system is the fact that the conditions for crystallization of the virus are well established. Hence, modified particles can readily be crystallised and the structure of the inserted peptide determined by X-ray crystallography, providing a unique opportunity for analysing how the mode of presentation of a peptide affects its immunogenicity. In fact, such a study was conducted by generating a series of chimeric CPMV constructions expressing the 14 amino acid NIm-1A epitope from human rhinovirus 14 (HRV-14), at different positions on the capsid surface [64]. Biochemical and crystallographic analysis of constructions expressing the NIm-1A epitope inserted into the βC′-βC″ loop of the S protein revealed that, although the inserted peptide was free at its C-terminus, it adopted a conformation distinct from that found when a similarly cleaved peptide was expressed in the βB-βC loop of the S protein. Adjustment of the insertion site within the βB-βC loop (by moving it one residue to the left) resulted in the isolation of a chimeric virus in which cleavage at the C-terminus of the foreign epitope was greatly reduced. Crystallographic analysis confirmed that in this case the epitope was presented as a closed loop. Polyclonal antisera raised against this chimeric virus had a significantly enhanced ability to bind to intact HRV-14 particles, compared with antisera raised against other constructs presenting the same epitope as peptides with free C-termini (the antisera raised against these constructions reacted strongly against HRV-14 VP1 in Western blots but bound poorly to intact HRV-14). In conclusion this study demonstrated that the mode of presentation of an epitope on a heterologous carrier can dramatically affect its immunological properties. For epitopes such as NIm-1A, which adopt a constrained structure in their native context (HRV-14 particles), presentation as a closed loop is probably essential for good mimicry. In contrast, the success of the chimeric CPMV particles displaying foreign antigens derived from parvoviruses, or the bacteria *P. aeruginosa* (described above), in stimulating protective immunity, probably stems from the fact that those antigens act as linear epitopes that are active even in a denatured form, because they fold on recognition by the corresponding antibodies and the entropic cost of their folding is not enough to prevent binding with enough affinity. Thus the accurate structural mimicry is not an essential requirement in those cases. Overall, the results of this study demonstrated the potential of the CPMV-based display platform for studying the relationship between peptide structure and immunogenicity.

Whereas no known plant virus can infect mammalian cells, the ability of the chimeric viruses to replicate in plants and spread in the environment, raises biosafety concerns associated with the transport, distribution, and administration

of these plant virus-based vaccines. One option to circumvent these problems is to render the chimeric viruses non-infectious using inactivation methods, such as irradiation with ultraviolet light or chemical treatments. However, these processes have to be carefully monitored as they risk altering the structural properties of the particles and therefore their immunogenic characteristics. Other possibility is to use VLPs derived from the plant virus capsid proteins instead of the infectious chimeric virus particles, for the presentation of the foreign epitopes [66]. Furthermore, since the VLPs do not longer need to be competent at packaging RNA or spreading within plant tissues, as the replication competent chimeric viruses do, this enables to extend the range of mutations (*i.e.*, more insertion sites and longer insertions) that it is possible to introduce into the capsid proteins, thereby extending the versatility of the particles as antigen display systems for vaccine development.

Insect viruses can also be used as platforms for antigen display (they do not replicate in mammalian cells). Flock house virus (FHV) particles are widely used for antigen display, attachment, and delivery in animals [63, 67]. FHV is a member of the insect virus family nodavirus. The capsid (30 nm) is composed of 180 subunits of the single coat protein, arranged with a $T = 3$ icosahedral symmetry. Several surface exposed loops are promising sites for insertion of foreign antigens. FHV particles can be used either as intact virions (and thus, replication competent in insect cells), or as VLPs derived from the expression of the coat protein in the baculovirus expression system.

An interesting application of the FHV-based display platform was the construction of chimeric FHV particles incorporating the extracellular domain of the anthrax toxin receptor 2 (ANTXR2) [67]. The foreign sequence was inserted into the two most exposed loops, at positions 206 and 264, on the FHV coat protein. The extracellular ANTXR2 domain contains 181 amino acids and adopts a compact Rossmann-like α/β fold. Importantly, the termini of this domain are separated by only 4.8 Å in the native structure, representing an ideal situation for insertion into a loop on a carrier protein. Indeed, the chimeric proteins assembled into VLPs. Cryo-EM and three dimensional image reconstruction confirmed that both types of particles displayed new density at higher radius. Pseudoatomic models of the particles generated by docking the X-ray coordinates of the FHV coat protein and ANTXR2 domain into the cryo-EM density revealed the expected differences in the geometric pattern in which the ANTXR2 domains are displayed on the surface. For insertion site 206, the ANTXR2 domains were clustered in groups of six at the twofold axes of the particle, whereas insertion at site 264 resulted in more even distribution of the foreign domain. The accurate folding of the inserted protein was confirmed by demonstrating that the chimeric particles functioned as an anthrax antitoxin *in vitro* and *in vivo*. Specifically, like native ANTXR2, the particles were capable of binding anthrax protective antigen (PA), which forms part of anthrax lethal toxin and edema toxin. Based on this ability, the chimeric particles could potentially be used as a therapeutic compound to treat anthrax infections. Interestingly, because of the differences in ANTXR2 display pattern, the two types of VLPs showed different potencies as an antitoxin, with chimera 264 having a lower IC_{50} for toxin neutralization than chimera 206. Computational modelling suggested that

this was because chimera 264 bound more PA molecules than chimera 206. Although both particles displayed 180 ANTXR2 domains, steric hindrance prevented full occupancy of these ligands. Instead, it was predicted that chimera 264 might bind 120–130 PA molecules whereas chimera 206 could only bind 60–90 PA molecules.

Given that the binding of PA to ANTXR2 is exceptionally strong (dissociation constant $K_D = 170$ pM) complexes formed between the chimeric particles and PA can be expected to be very stable. This prompted immunogenicity studies based on the assumption that polyvalent display of PA would induce a more potent immune response to this antigen than monomeric, recombinant PA, which is being developed as a second generation anthrax vaccine. Indeed, rats survived lethal toxin challenge 4 weeks after a single immunization with the VLP 264-PA complex, whereas animals injected with an equivalent amount of recombinant PA died. This result reflects rapid production of neutralising antibodies in the absence of an adjuvant, two key goals for the development of an improved anthrax vaccine. The chimeric FHV particles thus, might serve a dual purpose in functioning as an anthrax toxin inhibitor and in forming a basis for development of a new anthrax vaccine.

21.7 Perspectives and Conclusions

VLPs are appealing as vaccine candidates because their inherent properties (*i.e.*, virus-sized, multimeric antigens, highly organised and repetitive structure, not infectious) make them suitable for the induction of safe and efficient humoral and cellular immune responses. Currently, there is a clear trend towards the establishment of VLPs as a powerful tool for vaccine development. VLP-based vaccines have already been licensed for human diseases (HBV and HPV) as well as for use in the veterinary field (PCV2), and many more vaccine candidates are currently in late stages of evaluation. Moreover, the development of VLPs as platforms for foreign antigen display has further broadened their potential applicability both as prophylactic and therapeutic vaccines.

Currently, main efforts in VLP technology are focused in the development of new VLP-based antigen display platforms for vaccine development. Structural characterisation of VLPs is an important requirement for this aim. Structural studies are mainly performed by advanced cryo-EM and X-ray crystallography, focusing on the comparison of VLPs composed of different numbers and combinations of structural proteins. The use of biochemical methods provides details on individual viral structural components, as well as insights into the structural basis of assembly, packaging and the interactions of VLPs with host components. Many studies are aimed at characterising the minimal requirements for VLP formation or contemplate prospects of modifying the original proteins without hampering the natural ability of these proteins to assemble into highly organised macromolecules. As a

consequence, modified structural proteins appropriate for assembly of multipurpose chimeric VLPs can be eventually designed.

The relative ability of diverse VLP types to induce the different branches of the immune response is influenced by a number of factors that are VLP-specific. Therefore, it appears unlikely that a single VLP platform will meet all the desired requirements. However, the continued parallel development of multiple VLP platforms will ensure that individual vaccines can be tailored appropriately to the type of immune response required in each case.

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Further Reading

Especially recommended for further reading are references [9, 13, 14, 17, 24, 26, 41, 42, 64, 65] *listed above*.

Chapter 22
Nanoscale Science and Technology with Plant Viruses and Bacteriophages

Alexander M. Bittner, José María Alonso, Marcin Ł. Górzny, and Christina Wege

Abstract Nanoscale science refers to the study and manipulation of matter at the atomic and molecular scales, including nanometer-sized single objects, while nanotechnology is used for the synthesis, characterization, and for technical applications of structures up to 100 nm size (and more). The broad nature of the fields encompasses disciplines such as solid-state physics, microfabrication, molecular biology, surface science, organic chemistry and also virology. Indeed, viruses and viral particles constitute nanometer-sized ordered architectures, with some of them even able to self-assemble outside cells. They possess remarkable physical, chemical and biological properties, their structure can be tailored by genetic engineering and by chemical means, and their production is commercially viable. As a consequence, viruses are becoming the basis of a new approach to the manufacture of nanoscale materials, made possible only by the development of imaging and manipulation techniques. Such techniques reach the scale of single molecules and nanoparticles. The most important ones are electron microscopy and scanning probe microscopy (both awarded with the Nobel Prize in Physics 1986 for the engineers and scientists who developed the respective instruments). With nanotechnology being based more on experimental than on theoretical investigations, it emerges that physical virology can be seen as an intrinsic part of it.

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Keywords Bacteriophage • Biophysics • Capsid • DNA • Drug delivery • Hybrid materials • Lithography materials • Microscopy • RNA • Nanoscience • Nanotechnology • Scanning probe • Template • Virus

Abbreviations

1D	One-dimensional
3D	Three-dimensional
AFM	Atomic force microscopy
CCMV	Cowpea chlorotic mottle virus
CP	Capsid protein
CPMV	Cowpea mosaic virus
DEP	Dielectrophoresis
eBL	Electron beam lithography
GFP	Green fluorescent protein
ORF	Open reading frame
SEM	Scanning electron microscopy
SPM	Scanning probe microscopy
STM	Scanning tunneling microscopy
TEM	Transmission electron microscopy
TMV	Tobacco mosaic virus
VLP	Virus-like particles

22.1 Introduction: Viral vs. Artificial (Synthetic) Nanostructures

Synthetic nanoscale structures are closely linked to the subject of *nanotechnology*. This field, often also identified with *nanoscale science*, deals with the production and manipulation of matter at the atomic, molecular, and supramolecular levels. It covers the length scales from 1 to 100 nm (in an extended definition 0.1–1000 nm). Nanotechnology provides a fundamental understanding of phenomena and materials at the nanoscale, with the aims of using structures, devices and systems that have novel properties and functions, due to their small size. It involves also controlling the structures, and integrating them into micro- and macroscale material components, systems and architectures. Within these larger and more complex assemblies, the control and construction of the underlying structures and components remains at the nanometer scale. A similar organization is found in cells, which operate on the microscale, but rely entirely on the interplay of nanostructures such as proteins. In extreme cases, the critical length scale for novel properties and phenomena may be <1 nm (*e.g.*, for the manipulation of single atoms in scanning probe techniques),

or >100 nm (*e.g.*, nanoparticle-reinforced polymers can exhibit features of >200 nm size, based on the local bonds between the particles and the polymer).

Nanotechnology is a very broad concept, which implies the application of fields of science as diverse as surface science, organic chemistry, molecular biology, semiconductor and solid-state physics, microfabrication, etc. A huge driving force for a proper technology is that materials can effectively be made to be stronger, lighter, more durable, more reactive, more porous, or more conductive, among many other properties. However, the concept encompasses not only very recent, but also some very well established processes and products. Hence, hundreds of everyday commercial products rely on nanoscale materials and processes, from paint to computer chips. Many more will follow, so nanotechnology is a key technology for the future, and governments have invested billions of dollars in research.

Focusing on nanotechnology on the molecular scale of 1–10 nm, very few products are developed, the probably best-known being based on gold particles. A crucial issue on this scale is the development of simple construction schemes for the mass fabrication of identical nanoscale structures, just like chemical reactions can build molecules. Conventional "top-down" fabrication techniques rely on demagnification of a given structure; this approach can be energy-intensive and wasteful: Many production steps involve depositing unstructured layers and then patterning them by removing most of the deposited material. Moreover, increasingly expensive fabrication facilities are required as the feature size decreases. The natural alternative to "top-down" construction is the "bottom-up" approach. In this case nanoscale structures are built from their atomic and molecular constituents by self-assembly. However, the "bottom-up" methodology is technologically not yet possible because the assembly processes are slow, faulty and in most cases not sufficiently well controlled. Organic chemists try to tackle this problem by developing ever more complex subunits that assemble into nanostructures; on the other hand, specific intermolecular interactions and tailored self-assembly come for free with natural building blocks, such as virus capsid proteins, which are able to spontaneously assemble, under the proper conditions, into virus capsids and virus-like particles (VLPs) (see Chaps. 2, 10, 11, 19 and 21). Therefore, virus structures can and are being used as templates for directing the self-assembly of materials, and as scaffolds for nanofabrication processes.

A completely different motivation for the biomolecular approach [1] is based on the prefix "bio": The very fact that biomolecules are molecules of life implies that they have numerous active functional moieties that interact in complex ways with their environment. This can be used for binding or for the synthesis of inorganic and organic substances. Chemically reactive moieties on the biomolecules, such as amines and carboxyl groups, can be exploited to attract and react with other molecules. Second, substrate-specific affinities (antibody-antigen, biotin-streptavidin, oligonucleotide base pairs) have been employed to assemble substances in a programmed position, to align structured materials in a specially designed pattern, and to conjugate biomolecular substances with each other. Third, the

enzymatic activity of biomolecules is utilized to decompose or generate organic and inorganic substances by stabilizing intermediates during reactions.

A variety of biomolecules have already been exploited for the preparation of nanoscale materials. Oligonucleotides are commonly used in bionanotechnology due to their hybridizing functions and their ability to create reconfigurable structures [2]. Peptides can be synthesized in the laboratory and conjugated with organic molecules while maintaining the biological activities of catalysis and specific recognition. Certain peptides are able to mineralize inorganic sources with functionalities that are dependent on the composition and the structure of the peptide. Proteins and their assemblies serve as platform for nanomaterials synthesis as well. Proteins display secondary molecular forces such as hydrogen bonding, electrostatic and hydrophobic interactions that play an essential role in scaffolds for the fabrication of nanomaterials with defined geometry. Viruses are another type of biological macromolecular complexes that may be used for those purposes. The application of animal-infecting virus species and derivatives thereof is centered mainly on biomedical problems such as the development of antiviral agents and vaccines (see Chaps. 20 and 21) (with some notable exceptions); reasons for the infrequent use of animal viruses in nanotechnology include the possible pathogenicity of complete viruses, their dependance on special cells for their propagation, lower yields, greater expense and, frequently, high structural complexity with outer lipid envelopes. In contrast, plant and bacterial viruses (bacteriophages) are suitable for a much wider range of nanotechnological purposes because they have several advantageous characteristics [1, 3]. Major features that make virus particles potentially useful for many nanotechnological applications include:

1. Viral particles possess precise nanoscale structure and dimensions, in contrast to artificial nanomaterials prepared by “top-down” approaches. Their size range, from about 15 nm up to >2 μm (in diameter or length), is unique for organic structures, and in some cases characterized down to atomic resolution. Synthetic colloids and polymers of comparable dimensions rarely show such geometrical and chemical precision.
2. Viruses can be found in a variety of shapes (most importantly, icosahedron-derived, *i.e*. ‘spherical’ symmetries, and helical shapes (tubes and filaments), but also bullet-shaped particles; see Chap. 2). For most types of plant viruses and bacteriophages, all viral particles are identical in size and composition (in some cases the capsid exists in more than one size or shape), with one or more nucleic acid molecules (DNA or RNA) packaged into a protein shell made up of numerous subunits of one or a low number of distinct protein types. (A few plant viruses also produce empty additional capsids, and some harbour an outer lipid layer).
3. Viral capsids exhibit constrained internal cavities that are accessible to small molecules, but often impermeable to large ones, providing opportunities for assembly and packaging of cargos. The exterior and interior interfaces of the capsid have been utilized for directing encapsulation and synthesis of both inorganic and organic materials. E.g., nanoparticles of transition metal

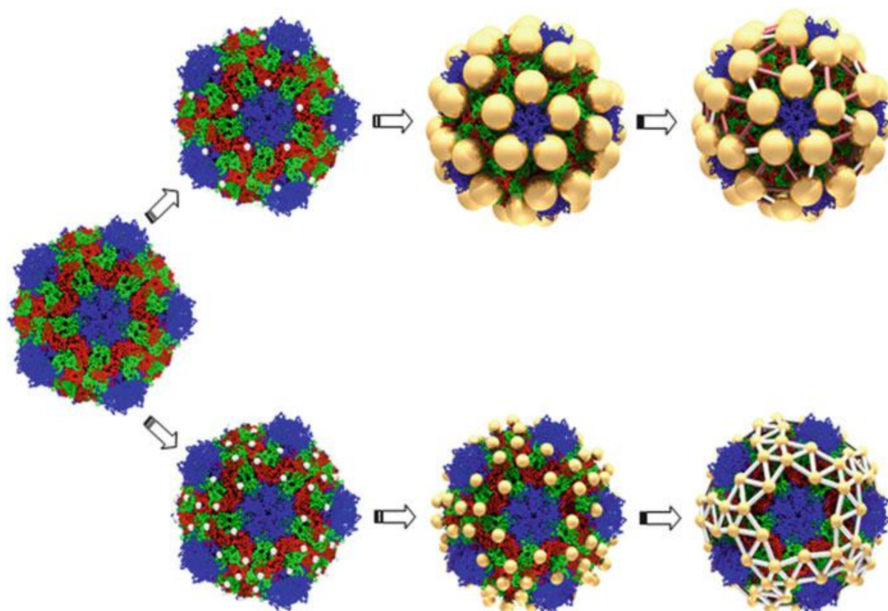


Fig. 22.1 Nanoparticles nucleating on nucleic acid-free CPMV. The *top row* shows a Cys (*white dots*) mutant of CPMV with attached 5 nm gold nanoparticles, and finally interconnected gold particles, bridged by organic molecules. The *bottom row* shows a double Cys mutant (two thiol groups per capsid protein), to which 2 nm gold particles bind, again to be interconnected (reproduced from [5] with permission)

polyoxometalates were nucleated on the interior interface of nucleic acid-free cowpea chlorotic mottle virus (CCMV), and gold particles on cowpea mosaic virus (CPMV) [4] (see Fig. 22.1). Other examples are medically relevant molecules, such as doxorubicin, which have been encapsulated through covalent attachment on the interior surface of virus-like particles from rotavirus structural protein VP6 [6].

4. Viruses represent the best examples of polyvalence and self-assembly processes. Several plant viruses do not depend on living cells for their self-assembly, but can be reconstituted from isolated components also *in vitro*. Viral particles have large surface areas, which permits the display of many molecules without concern of steric hindrance. Functional groups are displayed in multiple copies on the coat (capsid) Protein (CP) subunits forming the viral capsids, and offer anchor points for biochemical interactions.
5. The high strength of capsid protein-protein interactions makes several types of virus particles very stable. They are usually more resistant toward changes of pH, temperature, ionic strength and solvent than standard proteins, and afford a broader range of conditions for their isolation, storage and use.

6. Virus composition and properties may be tuned by manipulation of the viral genome. Exogenous oligopeptide sequences can be inserted with standardized mutagenesis protocols [7] (see also Chap. 21).
7. Mass production of viruses or their components may be easily achieved by growing them in infected hosts or in heterologous *in vitro* production cultures [8, 9] (plants, cultured insect, yeast or bacterial cells) with a yield in the range of 0.1–1 % by weight for certain viruses in suitable production systems. Moreover, viruses exhibit unique densities, making purification steps simpler and faster than those required for most proteins, and thus adjustable to large scale.

From a rather biochemical focus on viral surfaces and their functionalization, the discussion will move to assembly of viruses and of materials (for which viruses are used as templates). The characterization techniques and devices are more based on physics, while biosensors combine all disciplines.

22.2 The Control of Surface Chemistry by Genetic Engineering and by Chemical Reactions

As outlined above and different from inorganic templates, the protein surfaces of robust virus and bacteriophage nanoparticles harbour an enormous potential for the site-directed introduction of novel functional groups and, concomitantly or subsequently, even complex activities [10]. This may (1) modulate the structures' electrostatic charge, adapting them to different technical environments; (2) add novel chemically addressable anchors for the deposition of inorganic compounds such as metal or metal oxides or the immobilization of larger molecules for catalytic or detection purposes; and (3) incorporate extended amino acid sequences functional by themselves, *e.g*. as capture moieties or enzymatic units. The different strategies will be explained and exemplified in the following, with the term 'virus' being used for both plant and bacterial infectious agents unless otherwise stated. Some arguments also hold true for certain animal viruses such as adeno- and adeno-associated viruses (see Chaps. 10 and 11).

22.2.1 Chemical Modifications Using Naturally Occurring Reactive Groups

Among the natural amino acid building blocks of proteins, those with polar, basic or acidic or chemically highly reactive side chains are of superior importance for technical applications of viral templates, if accessible on their outer or interior surfaces – depending on the intended use. Cavities inside viral capsids are frequently exploited as 'casting moulds' to shape and protect inorganic deposits. Nanowires or granules can be formed inside tubular virions such as tobacco mosaic

virus (TMV), or in the shells of spherical (or quasi-icosahedral) viruses, *e.g.* CCMV, red clover necrotic mosaic virus, CPMV or brome mosaic virus, respectively. Additionally, hollow viral protein structures, with or without nucleic acid content, are explored for their potential to serve as vehicles ('capsules' or 'cages') for the targeted delivery of different substances to specific sites both in technical and biological environments (see Sects. 22.6 and 22.8). These applications frequently depend on the charge of the interior protein surfaces, providing attractive electrostatic forces and redox conditions, which retain or precipitate materials of interest inside the nanocontainers. Some strategies also involve direct covalent linkage of target molecules to chemically addressable groups in the viral container, predominantly amino, thiol, and carboxyl functions (see below). Similar prerequisites exist if viruses are applied as 'positive templates', *i.e.* proteinaceous carrier complexes for the fabrication of coated hybrid nanorods, filaments or beads. Depending on the molecules to be exposed on the viral backbones, either non-covalent interactions or chemical binding may be selected to interconnect the viral and the functional component. These two essentially different strategies are suited to mediate hierarchical assembly of virus-containing complex architectures and materials, and to integrate viruses and their derivatives into technical devices (see Sects. 22.4, 22.7 and 22.8).

Several naturally existing viral scaffolds of different shapes have been employed successfully for the attachment of inorganic, synthetic or biological molecules to their outer capsid or inner cavities. The reductive (electroless) deposition of metals and metal oxides from ionic precursor salt solutions, for example, depends primarily on the local chemistry of the capsid protein surface. It came out to work well with different, readily available viral templates. Similarly, mineralization of distinct viral capsids with silica coatings succeeded without a need for specifically addressable nucleation sites. Selective and direct covalent fixation of compounds, however, relies on the presence of readily accessible anchor groups. Various viral templates offer amino functions of lysine (Lys) residues, which can be conjugated efficiently with target molecules *via* alkylation or acylation, *e.g.* using N-hydroxysuccinimidyl ester or isothiocyanate derivatives. Thiol groups of cysteine (Cys) are well-established reaction partners for the coupling of sulfhydryl-carrying molecules or alkylation with maleimides [11, 12], but not frequently exposed on viral capsids. Carboxyl functions of acidic amino acids (aspartic and glutamic acid, Asp and Glu) are less attractive reaction partners, since the respective coupling procedures can suffer from the presence of an aqueous environment, which is typically preferred with viral particles, though not essential in any case. Carboxyl derivatization typically makes use of an activation step involving EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) and subsequent reaction of the resulting intermediate with primary amines to form an amide bond. A few other amino acids found on viral capsid surfaces as well as post-translational carbohydrate modifications linked to protein or lipid components of special viruses are, in principle, directly addressable by conjugation techniques as well, but are by far less relevant for straightforward chemical functionalization of virus templates. Finally, aromatic tyrosine (Tyr) side chains can be exploited for sophisticated coupling chemistry "beyond the labeling kit", such as

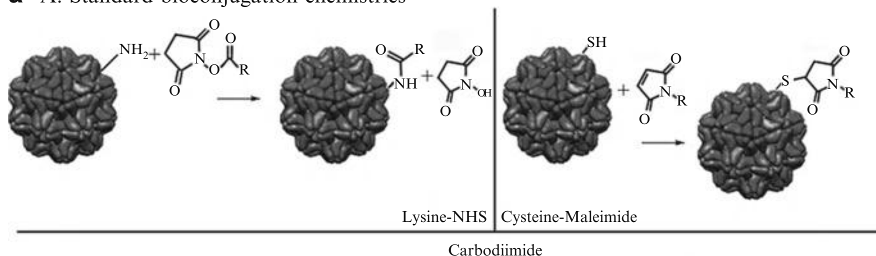
covalent diazonium linkage, but are more frequently used for intermediate chemical conversions preceding bio-orthogonal coupling (see below, and Fig. 22.2a for most common conjugation reactions to viral proteins). In summary, some natural anchor groups are found and easily used on a number of technically and scientifically interesting viral scaffolds, but many routes of research and development demand additional selectively addressable attachment sites for functional molecules, which need to be generated by biochemical or genetic engineering, respectively, as explained in the following.

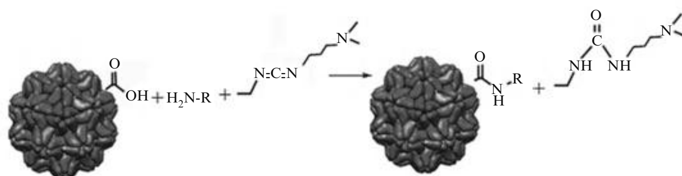
22.2.2 *Biochemical and Biotechnological Engineering Conferring Novel Addressability*

The first and relatively fast biochemical approach to viral capsid functionalization transforms surface-exposed side chains or groups accessible at the ends of CP subunits into advantageous functions. Here, besides Lys and Cys, Tyr with its phenolic hydroxyl group is among the most attractive target amino acids, since it is amenable to both oxime condensation and 'Click' chemistry. The respective two- or multistep reactions equip viral scaffolds with anchor groups or 'handles' accessible to selective, covalent and bioorthogonal coupling mechanisms, which do not affect the integrity of the targeted biomolecule. The most prominent examples are the copper-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) 'click' reactions, by use of which a large variety of distinct compounds ranging from fluorescent dyes to complete proteins has been interlinked with viral templates. The second experimental route towards a targeted integration of novel functionalities into virus-like particles is gene technology-based protein modification, that adds numerous further degrees of freedom to the development of virus-based tools. Although it is by far more time-consuming and might bear a substantial risk of failure, genetic engineering has proven the most flexible instrument for equipping viral shells with a multitude of distinct components, by itself or in combination with chemical coupling. Thereby attached functions include recognition and reporter elements as well as catalytically active units (see below). The respective approaches comprise both replacement and insertion of CP amino acid residues to modify local charges, to introduce chemically addressable sites (as described above), and to add peptides or extended protein domains. The following paragraphs give a short overview on essential molecular biology strategies underlying the genetic tailoring of viral capsids or virus-derived nanostructures.

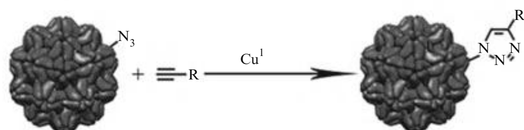
Prerequisite is the availability of the full genetic sequence information of a robust and suitably shaped template virus, or at least of its structural components if they can be assembled to VLPs outside the natural host. Typically, purified viral nucleic acid~~s~~ genomes or cDNA copies thereof have been cloned into bacterial plasmids (see Fig. 22.2b) or other vectors by biotechnology methods involving enzymatic modification, separation, ligation, and amplification steps. After

a A. Standard bioconjugation chemistries

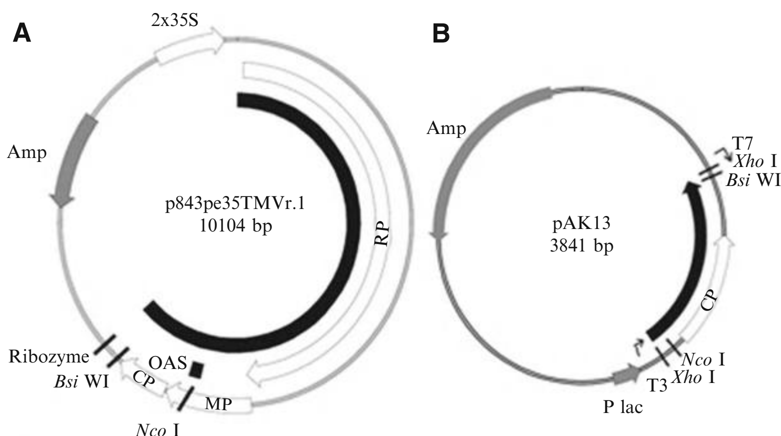




B. Copper catalyzed azide alkyne cycloaddition (CuAAC)



b



c

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wtTMV  (1)   SYSITTPSQFVFLSSAWADPIELINLCTNALGNQFQTQQARTVVQRQFSEVWKPSPQVTV
6xHis  (1)   ------------------------------------------------------------
E50Q   (1)   -------------------------------------------------Q----------

wtTMV  (61)  RFPDSDFKVYRYNAVLDPLVTALLGAFDTRNRIIEVENQANPTTAETLDATRRVDDATVA
6xHis  (61)  ------------------------------------------------------------
E50Q   (61)  ------------------------------------------------------------

wtTMV  (121) IRSAINNLIVELIRGTGSYNRSSFESSSGLVWTSSPAT
6xHis  (121) -(N)----------------------------------HHHHHH
E50Q   (121) --------------------------------------
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Fig. 22.2 Modification of virus capsids. (**a**) Common bioconjugation reactions: (A) addressing amino, thiol, or carboxyl groups (EDC – 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide); (B) bio-orthogonal coupling by ‘click’ chemistry on azide-modified amino acids (*e.g*. Lys, Cys or Tyr), reaction of cargo “R” bearing an alkyne (Adapted from [13]). (**b**) Genetic maps of: (A) a plant-infectious TMV plasmid; (B) a small intermediate construct used for mutagenesis of the TMV CP open reading frame; (**c**) alignment of three types of TMV CP amino acid sequences produced in plants: wtTMV (wildtype), 6xHis (C-terminally extended by hexahistidine peptide), E50Q (affecting TMV tube stability). For details, refer to [14]

sequencing has verified the identity of the genetic material, either whole viral genome clones are constructed and multiplied *via* plasmids adapted to infect suitable production hosts (*i.e*. plants or bacteria), or partial sequences encoding the viral CPs are inserted into protein production plasmids (in cases where capsids can be formed in the absence of infectious viral nucleic acid). Most viruses accessible that way form stable virions of relatively low complexity, some of which can also exist as empty shells or assemble *in vitro*. Their genomes are rather small, and for infectious clones, transmission methods for the propagation in plants have been developed, which circumvent the problem that most viruses naturally depend on certain insect, fungal or other vectors for being spread into new plants. Capsids which can be reconstituted *in vitro* may also be assembled from proteins produced in heterologous cell cultures (*e.g.,* bacteria or yeast). While the self-assembly of spherical or quasi-icosahedral viruses mostly does not allow extensive changes of the outer capsid dimensions, tubular or filamentous plant viruses can be shortened *in vitro*, and elongated both *in vitro* and *in vivo* by means of altered encapsidated RNA, which determines the length of the final nucleoprotein particle. Filamentous bacteriophages, though, usually cannot assemble outside cells and thus offer less possibilites of modifying their overall shape. The major aim of genetic engineering approaches, however, is to alter the surface chemistry of the resulting nanoparticles. In this regard, filamentous phages such as M13 or fd harbor superior degrees of freedom in comparison to most plant viruses: They are composed of typically five different proteins encoded independently on a small genome, all of which can be genetically tailored in parallel to change domains at both ends of the viral particles and their longitudinal outer surface selectively. For plant viruses, comparable numbers of different genetically programmed modifications, carried out at pre-determined sites on a single particle, have not been achieved so far. Notwithstanding, *in vitro* particle reconstitution from different types of protein monomers are distinct opportunities of many plant viruses.

Specific alterations of viral CP subunits are achieved by targeted modifications of their nucleic acid template, *i.e*. the CP open reading frame (ORF) mediating the proteins' expression inside host or cultured production cells (see Fig. 22.2b for an example of a plasmid used for modifications of the TMV CP ORF). Site-directed mutagenesis by means of enzymes and chemical nucleic acid synthesis is applied to exchange, insert or remove nucleic acid codons for amino acids of choice. A profound knowledge on the viral shell structure (see Chap. 2) and molecular determinants of assembly and stability (see Chaps. 10, 11 and 13) is advantageous to delimit 'permissive' domains of the targeted CP sequence, allowing an insertion or attachment of foreign residues at useful sites without abolishing the viral assembly competence. Furthermore, changes in the overall isoelectric point or in locally important side chains may affect or impede capsid formation as well (in some cases involving indirect effects, *e.g*. by abolishing post-translational CP modifications promoting structural integrity, or by provoking defense reactions of the plant host inducing death of infected cells). In summary, specific alterations of viral shells are possible in many cases, but major changes of charge and structure as well as extended insertions can go along with a risk of failure and may need a series

of constructs to be tested in parallel. Successfully modified capsids, however, may then efficiently and stably accumulate in bacterial, yeast or plant cultures, yielding countless nanoparticles for numerous purposes.

22.2.3 Engineered Functionalities

Point mutations and engineered insertions of individual amino acid moieties in viral CPs typically serve as target sites for the post-assembly linkage of diverse functions to capsid scaffolds, or the localized materials deposition by distinct chemical and biochemical approaches. Peptides of up to 15 amino acids and certain relatively short protein portions often serve as anchor sequences for the interconnection with further molecules; they can be fused to ends of CP monomers, which are freely accessible in- or outside the assembled particles, or they may be integrated into surface-exposed protein domains not suffering from structural alterations. Such capture and anchoring sequences are called 'bioaffinity tags' if they mediate non-covalent specific binding of small or complex ligands, including molecules designed to serve as adaptor or linker to another layer of distinct compounds or nanostructures. Typical examples are glutathione-S-transferase portions (binding glutathione), biotin-mimicking peptides (binding streptavidin), or tetra- up to hexahistidine tags (complexing Ni ions which bridge to other molecules). Though bioaffinity tags are widely applied and in many cases efficient anchors, their non-covalent linkage to the target and sometimes poor specificity can be problematic. Alternatives lie in targets ('haptens') of commercially available antibodies which may therefore be employed as 'glue' between viral shells and any other structure or surface fashioned with the respective antibody species. Finally, advanced multi-step protein interconnection strategies lead to site-directed covalent coupling between either two proteins or a protein and a non-protein partner. They involve specific enzymatic transfer reactions addressing chemical or genetic modifications of the target protein, but have not been tested extensively with viral derivatives yet. Due to the advantageous selectivity, biocompatibility and stability of the respective coupling procedures it may be anticipated, though, that they will find their way into virus-centered nanotechnology soon.

In addition to moieties mediating an attachment between viral and technical support or cargo components, respectively, protein portions with specific biological or reporter activities are among the most sought-after constituents of nanovirologists' toolboxes. Viral shells equipped with single or multiple types of functions are considered promising high surface-area carriers for sensing and delivery purposes in both technical and medical contexts. Proteins can realize major contributions to all these applications since they may serve as detector, transducer and effector molecules, depending on their origin and, optionally, subsequent optimization. Proteins can function as environmentally sensitive fluorescent dyes; catalysts for a nearly unlimited number of biochemical transformations; highly selective capture structures for diverse targets with recognition abilities even for unique

oligonucleotide or oligosaccharide sequences; they can induce light emission, deposition of mineral crystals, undergo active motion or modulate the viscosity of natural and technical materials. These and countless further capacities of proteins may confer novel abilities to technically useful nanoparticle preparations and nanostructured 'intelligent' or 'smart' materials. In conclusion, gene technological modification of viral templates is useful for fusing proteins of interest or active domains to viral CP subunits, and will increasingly be used.

22.2.4 Specialities Worth Mentioning: Towards Synthetic Biology and Biosafety Approaches

Among the distinct goals pursued by researchers in the field of virus-based nanotechnology, some deserve special attention since they either have yielded routinely applicable methods already, or are questioning widely accepted limits of feasibility. An exceptionally well-established and powerful technique exploiting viral scaffolds is the method of phage display. Initially described in 1985, the exposure of foreign peptides or protein fragments on pre-determined domains of filamentous bacteriophages (see also Sect. 22.2.2) has been developed into versatile selection systems for amino acid sequences with desired, adapted or even novel characteristics. *In vivo* or *in vitro* mutagenesis changes the genetic information underlying oligo- or polypeptide stretches linked to the surface of filamentous phage particles. When the mutations are – in certain limits – stochastically applied, random peptide sequences are generated, which alter the population of infectious phage units within a bacterial production culture. Alternatively, a library of distinct peptide-encoding sequences may be inserted into a starting batch of phages multiplying in a bacterial cell culture. The resulting viral nanoparticles are then harvested from the culture medium and tested for properties of interest, conferred by the physically linked foreign amino acids. Favorable phages selected *e.g.* by their binding to certain substrates are extracted from the remaining population (called bio-panning) and used to start a successive round of selection. Serial selection stages will enrich most suited phages, here with high binding affinity to the substrate of choice, and thus reveal an optimized genetic information for a peptide with the desired trait.

Numerous variations of this basic method have been developed and the identified peptides tested for applications *e.g.* in the fields of enzyme design, aptamer-mediated external gene regulation, and novel affinity reagents. One can not only exploit the selection capacity of phage-based systems, but also its filamentous backbone structures for the construction of novel process materials for industrial applications. Phage display may therefore be regarded one of the pacesetters of 'synthetic biology' approaches. Another strategy in synthetic biology also creates artificial virus-like protein assemblies: Novel protein shell structures were designed on the basis of known interaction domains of plant virus CPs, exhibiting

altered subunit arrangements, sizes and/or reduced structural complexity. Such 'nanocontainers' can either assemble from single or multiple monomeric building blocks directly inside the protein expression host transformed with corresponding genetic constructs, or may be fabricated *in vitro* from purified protein species. Finally, it is worth mentioning that plants have also been exploited for the heterologous production of empty and genetically tailored, non-infectious particles of human viruses, thus excluding any risk of animal cell-derived contaminants to meet strict biosafety regulations. Those particles are tested for applications mainly in the fields of diagnostics and therapeutics, with approaches comparable to those described for plant and bacterial viruses before.

22.3 Modification of Viruses with Functional Material

The driving force to create new and more complex nanostructures is on the one hand technology, *e.g.* scaling-down of computing, memory storage, and sensor devices; in other words, more functional elements can be placed in the same volume, as exemplified by the ever decreasing size of transistors and interconnects in computer chips, and by magnetic recording bits, but also by many biosensors. The usefulness of viruses relies on their complex and well tunable surface chemistry (see Sect. 22.2).

A typical application example for a functional solid material [15] is the design of superparamagnetic nanoparticles: This requires ferromagnetic matter cut to such fine bits that the magnetic moment can be switched by thermal activation, *i.e.* it fluctuates spatially. It is as intriguing as useful that typical sizes of virions are just above this superparamagnetic limit (a few nm up to about 20 nm size). Such particles, suspended in liquids, can be directed by an external magnetic field focus, which is useful for marking tumors. On the other hand, an oscillating magnetic field (with a flux of the order of 0.01 T, and a frequency on the order of 100 kHz) induces highly localized heating, which can destroy tumors. It is obvious that the exact size, shape, and chemistry of the particles is crucial. Spherical virus shells, similar to apoferritin, offer a much higher definition than particles synthesized by standard methods, and excellent biocompatibility.

As in this example, most functional materials require metal compounds, and so the construction of the nanoobjects is based on standard inorganic chemistry of metal ions, such as precipitation and redox reactions (Table 22.1). One interesting aspect is the interface to the virus: First, the usual metal ligands are incorporated in the virus surface. Here amine and thiol groups, often genetically engineered, are most popular, but also hydroxy groups can work very well. Second, a future nanotechnology (in a strict definition) could be built very elegantly on a combination of biology with chemistry and nanoscale physics [16]. The key is the combination of biochemical synthesis methods with inorganic methods. The advantage of employing virions or viral capsids as scaffolds is on the one hand a better definition of the nanostructures, compared to template-free syntheses; on the other hand,

Table 22.1 Examples for chemical deposition reactions of functional materials on viruses in aqueous suspension

$Au(III)_{ads} + 1.5\ BH_3 + 1.5\ H_2O$	→	$Au + 1.5\ BH_2(OH) + 3\ H^+$ (ads. and chem. reduction)
$Ag(I) + e^- + h\nu$	→	Ag (photoreduction)
$Ni(II) + BH_3 + H_2O$	→	$Ni + BH_2(OH) + 2\ H^+$ (electroless deposition, catalyzed by Pd and Ni)
$Si(OC_2H_5)_4 + 2\ H_2O$	→	$SiO_2 + 4\ C_2H_5OH$ (hydrolysis, sol-gel procedure)
$Ti(CH_3)_4 + 2\ H_2O$	→	$TiO_2 + 4\ CH_4$ (repeated; atomic layer deposition)
$Zn(II) + 2\ OH^-$	→	$ZnO + H_2O$ (catalyzed by Pd in presence of nitrate)

principally superior methods such as synthesis from small precursors (*e.g.* from polynuclear metal complexes) or even by manipulation of each single atom are far from being practical. A big challenge is developing recipes and especially general rules, such that known inorganic material synthesis methods can be tranferred to virus interfaces.

Organic coatings are quite unusual since the biochemical functionalization of the viral surfaces can be used to obtain a huge variety of chemical groups. However, in some cases simple or fast methods are searched for, such as coating with a polymer. Although the van der Waals forces between a polymer chain and a virus are huge, binding polymers is based on short-range chemical forces, such as hydrogen bonds. Not only a polymer synthesis, but any other synthesis at viral interfaces, requires careful balance between van der Waals, chemical, and electrostatic forces [17] (solvation *e.g.*) – exactly the same "colloid chemistry" that is required to bind nanoparticles to viruses, or viruses to viruses. In fact, some virus systems (TMV and fd phage) have contributed themselves significantly to the development of colloid chemistry since they offered the required submicro size, and defined surface charges.

Turning to the future, the highly complex chemistry of the virion surface allows building nanodevices with various, even multiple functions and physical properties. However, multifunctional nanoparticles are quite rare, and very few examples are known for virus templates. The decisive advantage of viruses would be their integration into micro- or macroscale solid devices: Here biochemical reactions on the solid surface, with their huge specificity, could make this process automatic (highly parallel "self-assembly", "bottom-up"). Possible solutions could be based on binding antibodies or parts of the virus (nucleic acid, capsid protein) to the surface. Such a strategy would work on the molecular scale, and has thus the potential to revolutionize nanofabrication. The standard nanofabrication is based on micro- or even nanoscale placement of the functional material, by lithography ("top-down" structuring – a widespread method is electron beam lithography, where the beam modifies a polymer layer pixel by pixel, see Sect. 22.5.3). This means that (nanoscale) devices have to be built sequentially, *i.e.* slow, while molecular recognition and self-assembly build complex molecules and nanostructures very quickly from smaller units, which assemble in a well-defined way, and on a smaller scale. The difficulty is to have a functional coating and also a biochemical function – the chemical groups should not interfere much with the function, and vice versa the coating must not obstruct the chemical groups.

22.4 Hierarchical Assembly into Complex Structures

Hierarchical self-assembly is characteristic of biological systems. It can be described as *formation of an ordered structure through a set of self-assembly steps*, *which decreases in strength* [18]. In other words, the term *hierarchical* refers to the situation where each self-assembly step is a guide for the next one. One of the better-known and understood examples of hierarchical self-assembly is the formation of TMV. Upon certain conditions, single units of a capsid protein interact with each other to form larger aggregates and discs (Fig. 22.3a). Assembly of a TMV is then initiated by a binding of an RNA origin of assembly loop to a two-layered disc (20S) comprised of 34 copies of a CP (Fig. 22.3b). Subsequent particle formation involves transformation of the 20S disc into a short helix and concomitant incorporation of the RNA strand between the CP layers. The resulting nucleoprotein helix is elongated bidirectionally *via* stepwise addition of further discs to one end of the growing nanotube, while the other end of the virus is completed by help of smaller CP oligo- and monomers. Such strategy of building up a capsid protein shell step by step around the nucleic acid has been observed in many other viruses with different geometries, namely polyhedra (see Chap. 12). More complex viruses often require much more complex tools, such as molecular motors, to pack the genetic material (see Chap. 12). Obviously, a very advanced nanotechnology would also make use of such nanoscale machines; here the focus is on rather simple cases, which can mainly be addressed as "self-assembly". It is important to note that in many cases the hierarchical assembly process is governed (at least in its initial stage) only by the information encoded in the protein itself, with the pH as the self-assembly triggering signal. This fascinating process, rather common in nature, is becoming an important experimental tool for the fabrication of complex mesoscopic structures, which often exhibit unique properties surpassing those of individual components.

One of the simplest methods of organizing particles into an ordered hierarchical structure is drying. By varying the virus and the salt concentration in solution, as well as the substrate surface properties, it is possible to tune the pattern formation during drying (see Fig. 22.3). Various structures can form in a capillary tube and on planar surfaces [19]. Stripe patterns or continuous films of filamentous viruses, of different thickness and orientation, are relatively easy to obtain with this technique from filamentous viruses. The ability to control surface arrangement of biomolecules promises new opportunities in sensing technologies or tissue engineering [20].

Another possible way in which viruses can form a hierarchical assembly is end-to-end interaction. In the case of TMV, the length of an individual virus is determined by the length of the RNA, but complete virions can arrange into a longer one-dimensional (1D) structure *via* interaction between proteins exposed at both ends of the viral rod, creating stacks of viruses (see Fig. 22.3). This ability is of utmost technological importance. 1D structures like nanowires or nanotubes are key components in a broad range of nano-applications. One interesting example is the synthesis of long, conductive polymeric composites of filamentous viruses, comparable to metal-coated structures discussed in Sect. 22.6. Such structures can have very high aspect ratios, and will probably find use in electronics, optics, sensing, as well as in biomedical engineering.

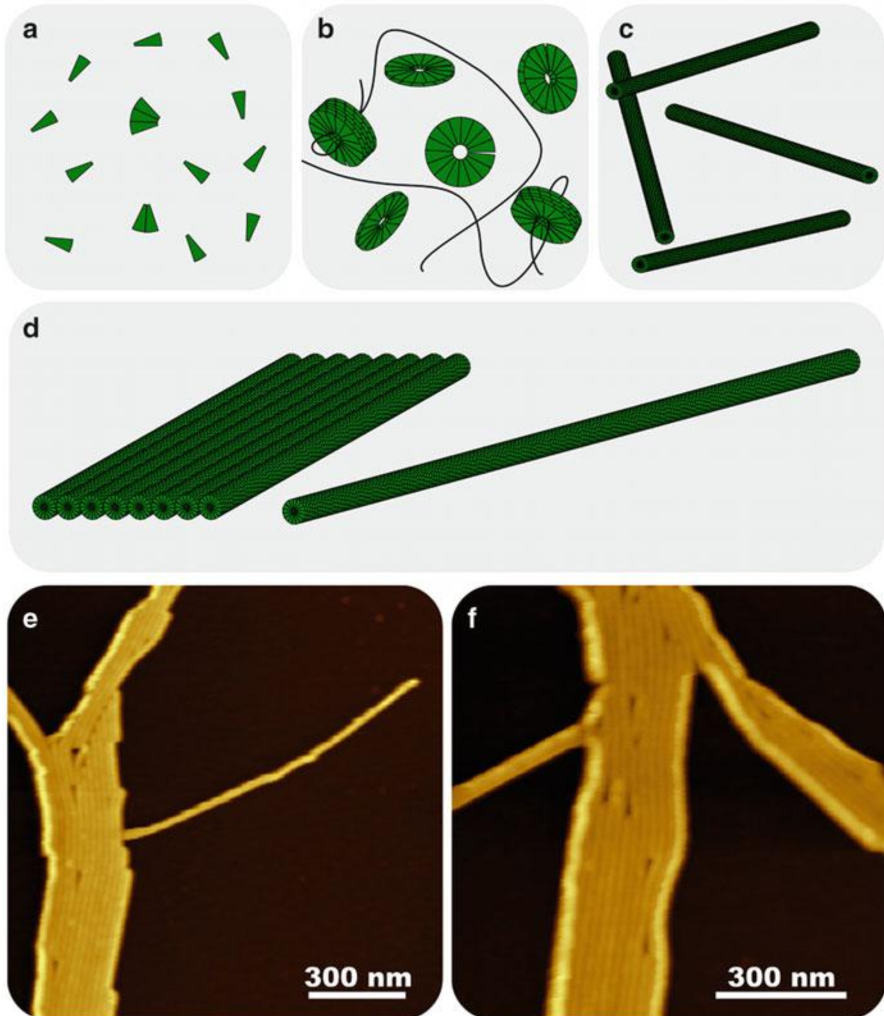

Fig. 22.3 Assembly processes of TMV. CP subunits form small aggregates (**a**), which assemble to form oligomers, rings and ring-stacks (**b**). An RNA origin of assembly positions itself within a two-layered 20S CP aggregate, which is transformed into a nucleoprotein helix, and recruits further rings and oligomers until the whole RNA is buried within the protein shell (**c**) *via* bidirectional assembly. For details, refer to Fig. 22.9. 1D structures can be extended by linear assembly when virus fibers attach to each other by end-to-end alignment, (**d**) *right*. Two-dimensional structures are analogous to nematic liquid crystals, but often imperfect, (**d**) *left*. Experimental examples (AFM) for TMV on a surface (**e**), (**f**) (image from M. Gorzny and S.D. Evans)

Rational assembly strategies for complex hybrid structures are quite rare: They can be founded on chemical and also on genetical modifications of CP surfaces, for which a set of standard methods is available. A bacteriophage can be programmed to selectively bind a chalcogenide nanocrystal at one of its ends, and metal particles on the capsid surface. Simple mixing of the phages with both nanoparticles results in such a complex structure, and further assembly can produce various architectures from 1D

to three-dimensional (3D). An example was investigated by Lee et al. [21], who exposed genetically modified M13 to ZnS nanocrystals, which spontaneously evolved into a hybrid bioinorganic film, extending to the macroscopic range (centimeters). While such combined approaches are typical of self-assembly on the laboratory scale, there is much thrust to use them for manufacturing electronic circuits in a truly “bottom-up” way.

Self-assembly strategies, as attractive as they may appear, intrinsically work in the microscopic world, based on interactions well below micrometers. Without highly sophisticated steering mechanisms, such as the ones nature developed in cells, they will not be of advantage for producing macroscale objects. Tackling the increasingly complex architecture of the nanoscale world may be much more rewarding. Typical examples are core-shell structures, for which ultrasmall chunks of organic or inorganic materials are coated by thin layers. Viral capsids can play the role of such layers, and since empty cavities are intrinsic properties of many plant viruses, the reverse approach is possible, and a range of mainly spherical virus shells have been filled with especially metal oxides and metals (see Sect. 22.3). This feature is of tremendous importance for many applications, out of which the most promising are drug delivery and guided growth of inorganic nanostructures like nanowires or nanoparticles.

When the viral shell is built up step by step around a given particle, the method is again a coating strategy. However, viruses allow for a much higher precision than chemical reactions or adsorption [22]: With the trick of binding the RNA assembly origin (RNA packaging signal, see Chap. 12) to a gold nanoparticle, simple mixing with CP subunits can result in assembly of virus-like particles around the gold nanoparticle. This artificial structure has the morphology and the chemical features characteristic of a virus. This strategy of employing the CP self-assembly processes to envelope an inorganic cargo could be used in many applications, *i.e*. drug delivery (see Sect. 22.8) or catalysis. The morphology and the chemical composition are thus more precisely tuned than possible with organic layers.

22.5 Nanoscale Analysis and Manipulation

Physics provides nowadays techniques that allow direct visualization, physical analysis and manipulation of single nano-objects including individual molecules. These techniques include atomic force microscopy (AFM; described in detail in Chaps. 8 and 18) and optical and magnetic tweezers (described in detail in Chap. 9). Here only a reminder of a few aspects of these techniques as applied to viral nanotechnology is provided. In addition, other physical techniques that are being used in viral nanotechnology are briefly described in this Section.

22.5.1 Scanning Probe Techniques

Scanning probe microscopy (SPM) techniques were originally developed for hard surfaces. Among these techniques, AFM (see Chap. 8 for a detailed description) has

proved to be an efficient tool to answer many fundamental questions concerning soft matter, including animal viruses, plant viruses and bacteriophages (see Chaps. 8 and 14). With AFM, viruses can be imaged in physiological media. Structural and morphological changes of the virus can be monitored in response to a change of the environmental conditions. Hence, this technique is a complementary tool for the immediate identification of viruses. Most important are contact and noncontact (mainly "tapping") AFM, with a vertical resolution of below 1 nm, although lateral resolution values below 10 nm are hard to reach. A large virus particle means a large vertical tip movement, which is usually not well compatible with high lateral resolution. However, special techniques allow to obtain more information: AFM can scan the interior architecture of viral particles, after systematically stripping away layers of their structure (see Chaps. 8 and 18). Plant viruses in crystalline form as well as single viral particles and bacteriophages can be visualized by this technique. Contact AFM is normally not suitable for scanning, but it can provide information about a variety of mechanical properties of individual viruses, including local adhesion and elasticity of capsids, although other methods like tapping or "jumping" modes (see Chap. 8) are generally more suitable. The Young's modulus and the Poisson ratio can be determined from nanoindentation experiments performed with AFM [23–25] (see Chap. 18).

In contrast, scanning tunneling microscopy (STM) is only useful for conductive and rather flat surfaces since it uses a conductive tip in close proximity (<1 nm), which however is not defined in shape (with the possible exception of some atoms at the tip apex). On poorly conductive objects such as viruses, the tip penetrates the object completely in order to reach the underlying surface. Even when the virus is coated by a thin metal layer, the relatively large height differences of up to tens of nm makes imaging difficult. Still, some reliable imaging conditions were found, most remarkably for usually nonconductive substrates such as glass, which acquire some conductivity upon hydration [26]. STM is also useful for detailed highly local current-voltage (I/V) measurements, which are however restricted to composites of viruses with metal or semiconductor coatings.

A further evolution of SPM is the dip-pen nanolithography. This technique uses an AFM tip as a nib, a solid substrate as paper and molecules with chemical affinity for the solid substrate as ink [27]. Plant virus and phage nanostructures can be fabricated by capillary transport of linker molecules from the AFM tip to a flat solid substrate, followed by incubation with the virus suspension. The linker molecules have to be bifunctional: One moiety binds to the surface, arranged by the tip in the desired micro- or submicro pattern, and the second one is employed for chemoselective attachment of the viruses. The control can reach the single particle level, when the pattern size and spacing is chosen appropriately.

22.5.2 *Optical Tweezers*

Optical tweezers (see Chap. 9) use a highly focused laser beam to provide an attractive or repulsive force to physically hold and move microscopic dielectric

objects. The basic principle behind optical tweezers is the momentum transfer associated with bending light. If an object bends the light, changing its momentum, the object undergoes an equal and opposite momentum change. This gives origin to a force acting on the object. In a typical setup, the incoming light comes from a laser which has a Gaussian intensity profile. When the light interacts with an object, the light rays are bent according to the law of refraction and reflection. The forces from such rays can be split into two components: the scattering force pointing in the direction of incident light and the gradient force coming from the gradient of the Gaussian intensity profile and pointing towards the center of the beam. The gradient force is a restoring force that pulls the object towards the center. If the contribution to scattering force of the refracted rays is larger than that of the reflected rays, then a restoring force is also created along the beam axis, and a stable trap will build up. Individual virions and oriented arrays of virions can be optically confined within volumes of a few cubic micrometers without damage, and manipulated in space with a precision of about 500 nm [28]. The very low and precisely tunable forces can be used to probe subviral structures, such as the packaging of DNA in phages [29] (see Chaps. 9 and 12).

22.5.3 Electron Beam Lithography and Photolithography

Nanofabrication techniques, such as electron beam lithography (eBL), permit the handling of single virus particles. eBL is a maskless lithography technique that uses a focused beam of electrons to pattern substrate surfaces covered with a polymeric film resist (tens to hundreds of nm thick). This technique is the simplest way to produce self-designed structures below 50 nm lateral feature size (even $<$10 nm can be reached). After electron bombardment, exposed or non-exposed regions of the resist are selectively removed in a developer solution. Negative resists form bonds or cross-links between polymer chains during e-beam exposure and turn out insoluble when developed, creating small areas of protruding structures. On the other hand, positive resists undergo bond breaking when irradiated, as a result exposed areas become more soluble in the developer. Therefore, small areas of recesses are formed. Depending on the desired design, positive or negative resist nanostructures are thus used to transfer material to the substrate, either by deposition of metals, or by etching. This technique has been widely applied for prototype integrated circuits, and for nanotechnology architectures. TMV is compatible with electron beam lithography processes and can be integrated in nanostructures made of positive and also of negative eBL tone resists. Viral particles maintain their biochemical functionality after fabrication steps, which was verified through selective immunocoating of the TMV [30] (see Fig. 22.4).

Photolithography is of much wider technical use. Here the structuring beam is ultraviolet (UV) light. Patterns are not formed by scanning, but by exposing a

polymeric film resist (the substrate) to UV light through a photomask, in one single fabrication step. The photomask is an opaque plate with holes (or windows) that allow light to pass through in a defined pattern. Such structures are the basis for microchip mass fabrication. The most advanced structures reach the deep nanoscale (<50 nm), but they require extreme UV and even soft X-ray optics, which are very rarely used in research laboratories. However, these methods are very suitable for arranging viruses, and as yet practically not explored.

22.5.4 Electric and Magnetic Fields

Manipulation of plant viruses can be based on the application of electric and magnetic fields. Best known is electrophoresis in porous media (gels) for particle separation; however, this is limited to the macro- and microscale. More suitable is dielectrophoresis (DEP), where viral particles move induced by the polarization effects in a non-uniform electric field (see Fig. 22.5). The non-uniformity (the field gradient) is induced by microstructured electrodes, and can thus work on the nanoscale. Indeed, DEP is known generally as a method to orient and to move colloidal particles, hence the application on viruses is straightforward. Movement is caused by the unbalanced force of the non-uniform electric field on the viral particle's induced dipole moment: one "end" of the dipole is in a weaker field than the other, causing the particle to be pulled electrostatically along the electric field gradient. Plant viruses as TMV and CPMV can be accumulated and oriented at microstructured electrodes using this technique [31, 32]. TMV presents a high polarizability, since it lacks an insulating membrane. Therefore TMV exhibits positive dielectric behavior (it is attracted to the electrodes) or negative (it is repelled from electrodes) depending on the frequency of the applied electric field. Likewise, CPMV presents positive dielectric behavior at low frequency of the applied electric field (2–3 MHz), and negative dielectric behavior at higher frequencies (18–20 MHz) [31].

In analogy to the electrical polarizability, also the magnetic susceptibility can be highly anisotropic. However, since this effect is based on the (very weak) diamagnetic moment, which is present in all matter, a rather large flux (1 T range) is required for orientation, and a large field gradient for movement. The orientation effect is well known from the alignment of nematic liquid crystalline and colloidal crystalline samples, especially for TMV and fd, which are staples in the research of liquid crystals [33]. Small-angle X-ray scattering is a typical detection technique. Fields and field gradients can be combined and varied in time to achieve a right-circular or left-circular orientation of TMV [34].

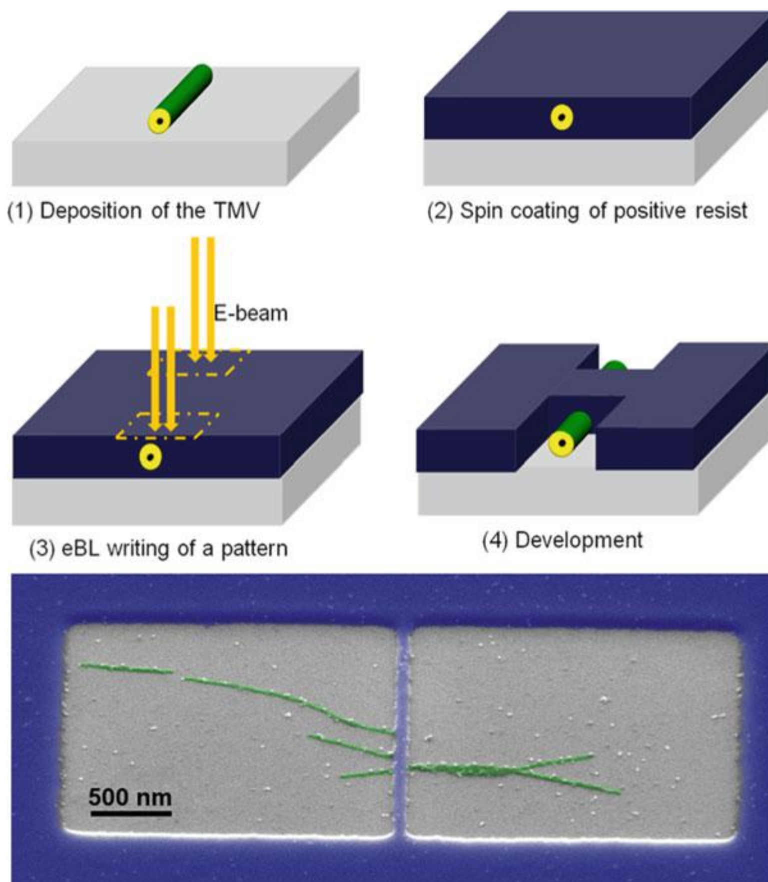

Fig. 22.4 Integration of TMV into typical electron beam lithography production steps. Typical nanofabrication schemes involve polymer coating, electron beam structuring, and selective dissolution of the irradiated polymer. *The bottom image* is a scanning electron micrograph that shows linearly assembled virus particles below a segmented rectangular polymer window produced in this way [30]

22.6 Viruses as Templates

A typical strategy in nanoscale science and technology is to employ a nanoscale object to achieve a certain function. The object – here a virus – provides proper size and shape, and a coating or filling bestows the function. The material, mainly inorganic, is difficult to tailor – that is why a template is required. In such strategy the virus template plays no biological role, so one could call it "inactive". The material on or in virions would then be "active", and tailored on the nanoscale. It might exhibit physical properties that are different from those of the bulk material, such as quantization of electrical conductance, (dis)appearance of

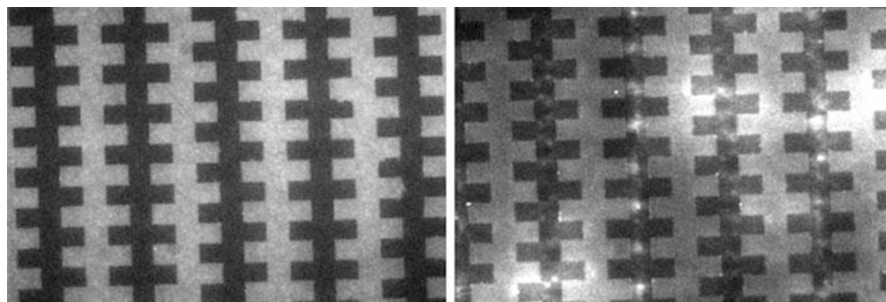

Fig. 22.5 Dielectrophoretic enrichment of plant viruses at microelectrodes. Switching on an alternating electrical field (20 V, 1 MHz) leads to the appearance of fluorescence (*white spots*) due to high local CPMV concentration. The frequency-dependent polarizability of the virus is at the origin of this phenomenon [31] (image from I. Ermolina)

ferromagnetic coupling, changes in light absorption and emission, or simply a high ratio surface atoms/bulk atoms. In this respect, inorganic structures (*e.g.* metals, metal compounds and ionic crystals) and organic/inorganic composites are often more attractive than organic molecules.

While nanoparticles are produced since many decades (and in some cases known for centuries), a relatively new aspect is the controlled fabrication of devices (see Sects. 22.7 and 22.8), and integration of nanostructures – both with nanometer precision. Linear structures (wires, tubes, chains of clusters) play a major role as spatial directors and as nanowire interconnects. The advantages of employing coated viral fibers, compared to conventional nanowires, are a better spatial definition of the nanostructures, and better defined functional groups. Moreover, principally superior ideas, such as nanowires that are defined to atomic detail, are not in reach.

A good example is the anisotropic shape of material on or in tubular virions [35], which can align the magnetization direction with the virion axis. The best known examples are gold-coted virions; in fact, the history of gold nanoparticle binding dates back to before 1939, when the first images of gold on virions were recorded [36]. The motivation was the shift of the optical absorption, resulting in deepening of the red color, used for virus detection. The specificity is too low for most uses, and it is more likely that this method will be useful to design highly defined plasmonic nanostructures. A third application example concerns electrical conductivity that was shown for Pt-covered TMV [37]. However, most modifications have as yet not found any application.

22.6.1 *Templates for Material in Viral Cavities*

Many RNA bacteriophages are tightly packed nucleoprotein complexes, with no internal cavities. Many DNA phages, some RNA phages and most plant viruses, in

contrast, have either a spherical cavity, which is filled by RNA or DNA, or an internal tubular channel, which is empty (filled by the surrounding liquid, and in dry environments likely by condensed water). Spherical viruses can in many cases be disassembled into protein and nucleic acid, and the pure proteins can be reconstituted into the empty shell, which should now also be filled by electrolyte (see Chaps. 10 and 11). Exactly this procedure is well known from a related nonviral shell, apoferritin. Apoferritin is filled with a range of oxides and metals, based on the chemical reaction types described in Sect. 22.3, and the same strategy can be used for spherical viruses.

A successful filling by solid material will depend on this reaction, and on the chemical functionalities present on the virus. It is crucially important to avoid any reaction on the exterior surface, hence the groups, their charges, their reactivities should differ as much as possible. It turns out that these conditions cannot be easily fulfilled, even for genetically engineered viruses. Moreover, the final material size is mainly well below 5 nm – template-free strategies cannot easily reach this range, so this could develop into a real chance for the technical use of viruses. However, as yet, only few examples of materials encapsulated in viruses are known. Some spherical viruses can be disassembled, the RNA removed, and reassembled. Ions can diffuse through pores in the shell to the interior cavity, and react in analogy to the examples described in Sect. 22.3. Iron oxide particles inside such a small cage are remarkably small, so small, that magnetic couplings accumulate to unusually small moments. This superparamagnetism is a very typical difference to larger particles, and very useful for ferrofluids (see Fig. 22.6).

Many filamentous viruses have a tubular channel. The concentration of certain amino acid moieties in the wall is so high that cations and some metal complexes show a preference for it, and materials can be grown inside. The frequent observation of small particles suggests a mechanism of nucleation, followed by rapid growth. When the material fills the channel, *i.e.* when it produces a wire, liquid is encapsulated in the virion [39]. This explains why a given channel cannot contain more than two wires. This reaction is best known from two-step electroless metallization. The yield and control are probably not yet sufficient to merit production of useful amounts of wires. Optical, electrical, and magnetic properties of the wires should differ hugely from bulk material, the main reason being the spatial confinement of the electrons.

22.6.2 Templates for Material on Viral Surfaces

In principle, the argument for material synthesis in a cavity can be reversed to achieve full selectivity for the external capsid. However, in most cases such thick layers of material are produced that a potential presence of it in a cavity has no influence. The exterior surface of virions is easily accessible, so a plethora of organic reactions (Sect. 22.2), adsorption, and of solid material synthesis are available (see Fig. 22.6). This is closely related to surface chemistry and surface

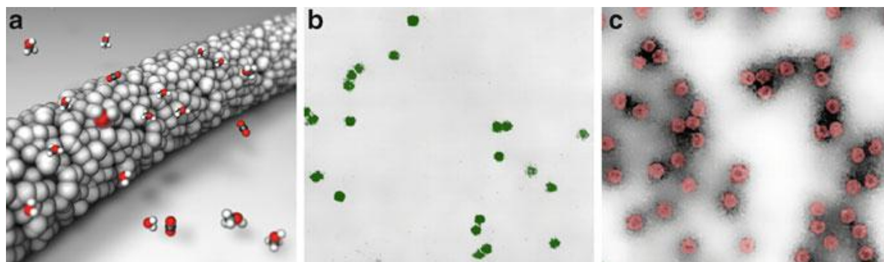

Fig. 22.6 Coating and encapsulation of inorganic materials. (**a**) Model of TMV coated by a layer of platinum nanoparticles. This structure can be used as electrode for fuel cells that oxidize organic compounds to carbon dioxide and water. (**b**), (**c**) Encapsulation of iron oxide in CCMV. Without staining, TEM shows only the high density core (**b**), with selective staining the whole particles is visualized (**c**) (reproduced from [38] with permission)

science, with the surface here being the virion. However, many methods (see Sect. 22.3) produce rough and thick (>50 nm) coatings, due to badly controlled nucleation or growth rate. Such structures can often be produced in simpler ways, hence growing materials on viruses might not be competitive. In some cases however, mainly for tube- or wire-like phages and viruses, the result is still useful because other ways of synthesis are complex. Magnetic coatings turn the template into a nanoscale magnet (Fig. 22.7), with uses in ferrofluids for advanced vibration damping. Ferrofluids are based on a much increased viscosity whenever a magnetic field is switched on; they require nanoscale magnets.

When viruses are assembled in parallel fashion, metallisation can produce a rather well-defined surface of high porosity. Based on this idea, various metals and oxides on M13 phage and on TMV have been tested as electrodes in nickel and in lithium ion batteries [41, 42] (see Fig. 22.8). Improved control of roughness and of parallel assembly can now open the way to use virions in electronic devices (Sect. 22.7).

22.6.3 Double Templating

Such systems have to be based on those discussed in Sect. 22.6.1. An additional reaction step, based on that described in Sect. 22.6.2 will now produce sphere-in-shell systems, or coaxial wire-tube systems. These can be very attractive since there are very few other methods to design matter in 3D so finely. For example, ferromagnetic cores are coated with antiferromagnetic shells to produce a coupling that can lead to exchange bias (asymmetric magnetic hysteresis curve) – this could be done much simpler with a spherical virus that is first filled in the core. There is a huge drive to applications such as ultrahigh density magnetic storage, where each particle would make up one magnetic bit. However, the required fabrication steps are as yet not known, and analysis methods on the required scale, *i.e.* <5 nm for the interior structure, are scarce. Indeed such small structures lead us to the limit of today's nanotechnology.

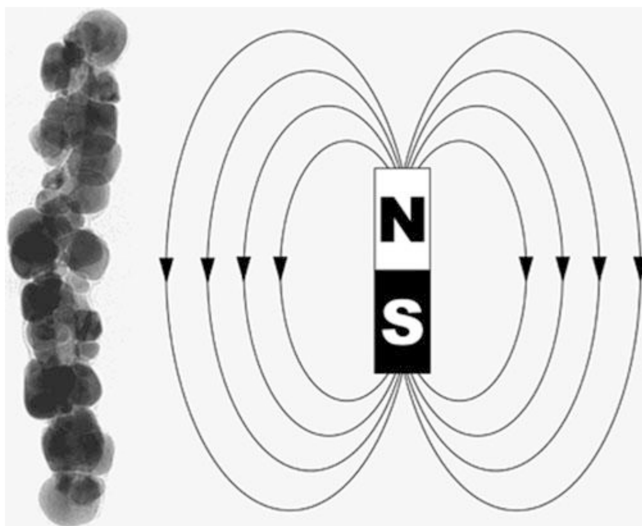

Fig. 22.7 Cobalt-plated plant virus as nanoscale bar magnet. Plant viruses, here two TMVs, can be coated by first adsorbing noble metal complexes (catalytic precursors), then reducing them and applying a metallization solution. Here cobalt coating was achieved [40]; such rather thick layers are ferromagnetic, hence a filamentous virus transforms into a nanoscale bar magnet (reproduced from [40] with permission)

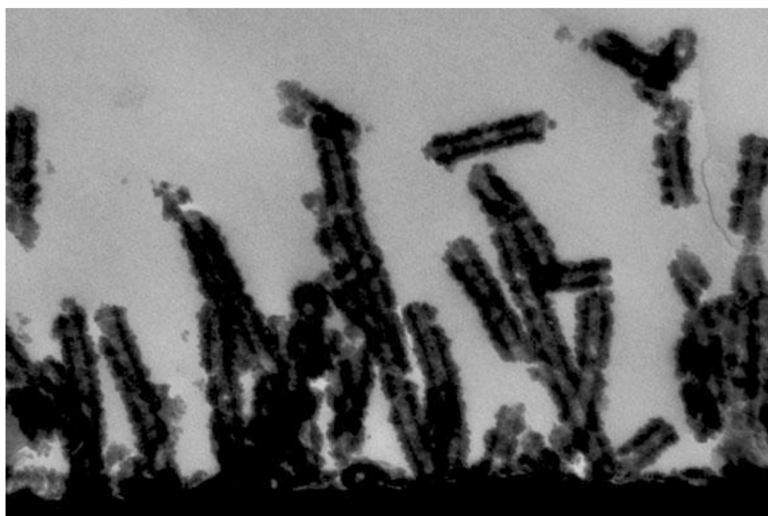

Fig. 22.8 Battery electrode made of nickel-plated TMV. The virus has to assemble standing-up on the flat substrate (*black*, at *bottom*), which is achieved by a Cys mutation. The virus layer is then treated with Pd(II) as catalytic precursor, followed by electroless deposition of a nickel layer. In this way, a highly porous surface is obtained [41] (TEM image from J.N. Culver)

22.7 Electronic Devices

Nanostructures made by using viruses as templates have been synthesized for over 10 years. Despite intensive research, most of the published work has concentrated on the synthesis. Indeed there have been only a few reports showing technological applications of virus-inorganic nanostructures. One can distinguish applications that require mass fabrication, *e.g.* ferrofluids and energy generation or storage, and those which require relatively small amounts, *e.g.* biosensors or nanowires (electrical connectors). The following paragraph we demonstrates these concepts with a few examples.

A good example of taking advantage of the virus morphology has been demonstrated by Royston et al. [41]: Engineered TMV assemblies, with cystein residues incorporated into the capsid protein, were vertically patterned onto gold substrates *via* gold-thiol (cystein) interaction. Nickel and cobalt were then deposited by electroless deposition. This layer of vertically assembled metal-coated viruses was subsequently used as an electrode in a nickel-zinc battery (Fig. 22.8). Tests showed that the incorporation of virus into the electrode increases its surface area and in consequence doubles the total electrode capacity. In a similar spirit, platinum-coated TMV has been tested as an anode material for a direct methanol fuel cell [37]. For this, TMV has been decorated with fine platinum nanoparticles by means of electroless deposition. During the growth, Pt nanoparticles interconnected with each other, thus creating a continuous and thin metallic shell around the virus (Fig. 22.6). Such hybrid Pt-TMV nanotubes formed a so called support-less catalyst, which in tests overperformed (higher surface area and stability) conventional, nanoparticle-based catalysts. Utilization of TMV-based catalysts is hence a prospect for reducing the amount of Pt (costs), and also for improving the cell efficiency. For microelectronic devices, viruses have not yet been used extensively. Some of the most impressive results were obtained with apoferritin, *e.g.* for floating memory [43]. Metallized virus-based nanostructures, especially 1D-like nanowires or nanotubes, are natural candidates for new types of electrical interconnectors. Electrical properties of virus-based nanowires/nanotubes were investigated by rather few researchers, but it emanates that conductive structures based on viruses can be exploited [44]. Metal/virus composites embedded in polymer films may even show memory effects [45].

Due to the size and linear morphology TMV has been found to have a profound effect on magnetoviscosity of a cobalt ferrite-based ferrofluid [46]. Simply by mixing a commercial ferrofluid with a TMV suspension one can observe the increase of magnetoviscosity. Moreover, the magnetoviscosity has been found to be less susceptible to a shear thinning (*i.e.*, a reduction of the viscosity with the applied stress) which is a desirable from the technological point of view. This new class of compound could potentially be used in micromechanical dampers.

Since viruses are nearly exclusively used as (advanced) scaffolds for active elements (nanoparticles or metallic layers), the design of a functional device based solely on a biological structure is especially challenging. An excellent example has been demonstrated by a Lee et al. [47]: Genetically engineered M13

phages with overexpressed pVIII N-termini (with a variable number of the negatively charged amino-acid glutamate) were allowed to assemble on the surface (see Sect. 22.4) of a gold film. Putting another gold electrode on the top creates a Au-M13-Au sandwich. Such structures exhibit a piezoelectric effect capable of producing up to 6 nA of current and 400 mV of potential capable to power up a small liquid crystal display.

22.8 Biochemical Detection Arrays and Targeted Drug Delivery

Besides their amenability to chemical modifications and materials synthesis or encapsulation, which typically result in bio/inorganic hybrid structures or conjugates with small organic compounds, the protein surfaces of viral templates offer unique opportunities for the fabrication of biochemically active 'smart' materials and arrangements with complex activities. As outlined in Sect. 22.2, selective capture or attachment as well as signal transduction capacities are amongst the technically most attractive functions of proteins. Their effective utilization in sensing or targeting devices, however, still poses a number of challenges. (1) First of all, the active sites of proteinaceous effectors need to be freely accessible to the target compounds. Furthermore, full reactivity often demands for conformational flexibility of the polypeptide chain. Both are best achieved by an exposed position of the functional unit on a suitable nanostructured scaffold in a controlled orientation. (2) Second, non-specific binding of the target molecules to the scaffold surface should be avoided, for which a different type of protein may be an ideal 'blocking' compound. A close vicinity of other proteins to the reactive one was also proven to generally promote and preserve biochemical activities. (3) Third, a high, but adjustable density of the active units on a polyvalent carrier structure with multiple immobilization sites is essential to ensure optimum functionality in different environments. (4) Finally, an efficient and targeted integration of the carrier into a device or a biological environment should be possible. Current protein-employing sensing arrays or targeting nanocapsules, however, usually cannot fulfill all of these demands and thus suffer from several limitations. While *e.g.* sterical hindrance can be reduced by fixing active protein units on technically fabricated nanopillars or networks of synthetic phases, this strategy alone is not sufficient to counteract adverse surface effects on both targets and active protein sites. An attachment of flexible linkers such as nucleic acid fragments or bioaffinity tags to pre-selected amino acid stretches of the bioactive unit can improve its reactivity due to controlled orientation and spacing to the support, but still needs additional measures against unwanted interactions with it. The immobilization reaction itself relies on the presence of suitable addressable groups on the carrier surface, which are lacking on typical array materials and thus are generated *e.g.* by additional coating layers. These should supply predictable reactivity for stable interconnections without negatively affecting protein or reactant integrity. Though numerous formulations of such 'adhesives' have been developed for the one- or two-step

fixation of proteins, their binding efficiencies and side reactions are still difficult to control in many cases.

Compared to assemblies on conventional planar, or advanced nanostructured synthetic substrates, peptide or protein ensembles exposed on multivalent viral capsid surfaces therefore have numerous advantages. Realized by means of biochemical linkage to pre-defined target sites, or by genetic fusion to selected amino acid portions of all or a subset of the viral CP subunits, they meet indeed all the requirements for highly active versatile arrangements listed above. In conclusion, virus-scaffolded protein arrays are most favorable and technically promising composite materials, applicable for both sensing and targeting purposes as explained in the following.

22.8.1 *Display of Capture and Targeting Functions on Viral Shells: State-of-the-Art*

Protein or peptide portions with selective affinities, presented on the surface of viruses or VLPs, offer numerous possibilities for their use in detection systems. Most compatible with existing read-out technology is an application of the viral templates as polyvalent adaptor phase between a technical support and the biochemically active units. The virus-derived templates thus will arrange, position and stabilize target-binding amino acid domains of one or more types in close vicinity to each other. While small peptides capturing or complexing ions, metals or simple compounds have been directly exposed on virus capsids by means of genetic fusion (predominantly on bacteriophages), larger functions catching compounds with *e.g.* medical or environmental relevance are mostly attached by chemical linkage or standard bioaffinity tags as described in Sect. 22.2. In this context, the typically employed proteins are antibodies or engineered derivatives thereof. Antibodies immobilized on spherical plant viruses such as CCMV or CPMV react for example with pathogenic bacteria or surface markers of distinct cell types (for these and numerous further references see [10] and [48]). In principle, also a direct *in planta* production of VLPs coated with extended capture domains or other additional protein fragments is possible, given that the genetically tailored fusion proteins assemble despite the foreign portions. Careful design of both the linking strategies between the viral CP and the non-viral domain, and molecular tools optimizing the production kinetics of the respective constructs have allowed to harvest functionalized chimeric nanoparticles from plant expression hosts at high yields. Stiff tobamovirus (turnip vein clearing virus TVCV) rods displaying antibody-binding fragments of *Staphylococcus aureus* protein A [49], flexuous potato virus X particles covalently coated with antibody derivatives directed against a herbicide [50] or spherical empty CPMV virus-like shells presenting GFP [51] may exemplify this most straightforward and thus economically promising strategy, which,

however, is less universally applicable than *ex-situ* linkage between viral shell structures and functional moieties up to now.

22.8.2 Perspectives of Virus-Scaffolded Detection Arrays

The utilization of accordingly functionalized virus-like nanoparticles in detection systems then might follow distinct approaches. Numerous protocols for the deposition of viral capsids on various useful substrates are available, controlling not only surface coverage, but also bonding strength, precise position and even orientation of the nanoparticles (refer to Sect. 22.4 for additional information). Advanced procedures allow for the fabrication of patterned and layered three-dimensional arrangements with blends of distinct and, where appropriate, interacting functionalities. These may include also conductive metal components allowing for electronic measurements [5, 52], see Fig. 22.9. The resulting arrays are high-performance capturing units which can serve as concentrator and indicator for specifically addressed targets. They may either be employed in enzyme-involving diagnostic assays such as ELISA (enzyme-linked immunosorbent assay) formats in combination with secondary binding partners *e.g.* for a colorimetric compound detection, or they can be integrated into sensor devices exploiting electrochemical or physical read-out technology to detect captured analytes, *e.g.* by the generation of electrical currents, changes in conductivity, surface plasmon resonance or direct quantification of adsorbed material by a quartz crystal microbalance. A coherent overview on analytical principles applicable for virus-based sensing has been published by Mao et al. [55].

Finally, three additional options to further improve sensing systems by way of viral derivatives should be noted. First, certain virus templates may not only be deposited on technical supports post-assembly, but may be grown from their building blocks “bottom-up”, *i.e. in situ* directly on attachment sites pre-defined by the local presence of an anchor molecule. This strategy was developed recently for TMV-derived nanorods on patterned polymer substrates (Fig. 22.9) [54] and may help to generate freshly functionalized carrier templates on demand, prior to their use even in difficult-to-access reaction chambers of nanosized future devices. Second, for increasingly diverse applications, planar detection microarrays are being replaced by ‘liquid’ or ‘bead arrays’ these days, with the selective binding reactions taking place on accordingly functionalized nanoparticles in suspension. Combinations of internal bead-specific labels (barcoding the affinity of the respective bead species) and flow separation technologies enable highly sensitive multiplexing approaches allowing simultaneous detection of distinct analytes. Since nanobead preparations can be densely equipped with viral templates as well, the resulting composite beads with their amplified soft matter surface-area and additional degrees of freedom for combinatorial activities on every functionalized bead are promising candidates for further improved liquid arrays. Third, the nanoparticulate nature of the polyvalent viral capsids themselves offers the striking opportunities to use them directly as array particles, or as signal-enhancing effectors in both conventional and novel detection

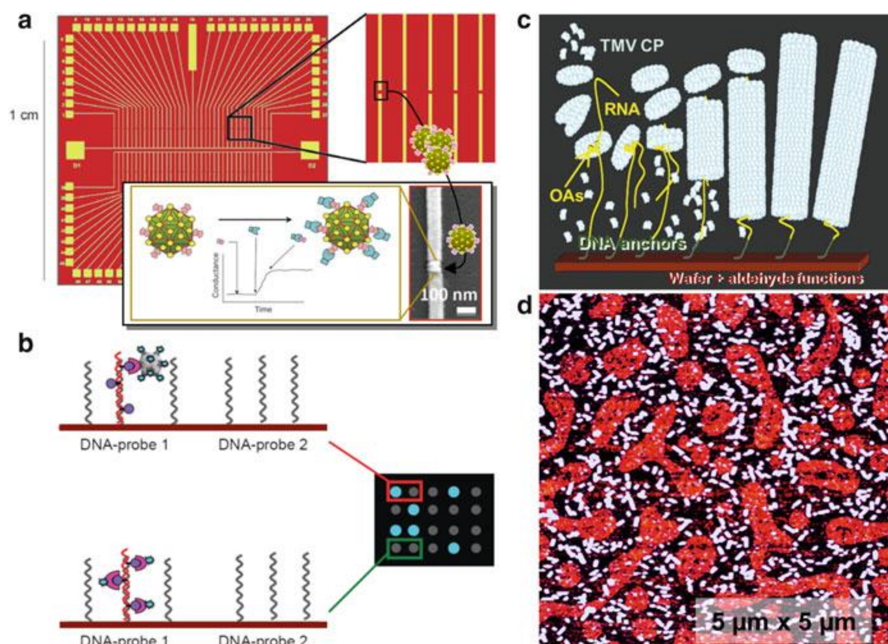

Fig. 22.9 Biosensing and positioning by help of viral particles. (**a**) Electronic sensor chip with many gold electrode pairs with common drain. Gaps contain dual-functionalized CPMV carrying *gold* beads and biotin (*rose*), specifically binding avidin and related proteins (*blue*), which results in conductance changes [5]. (**b**) CPMV exposing streptavidin (SA, *pink*) and numerous fluorescent Cy5 dyes (*blue*) as signal enhancers in microarray detection technology. DNA-probe 1 (but not 2) is targeted by a biotinylated DNA (*red*). Its biotin molecules (*violet circles*) are captured by SA, which in 1:1 conjugation to Cy5 (*bottom*) does not yield a detectable signal (*right*), while it does on the viral 'Cy5 multiplyer' (*top*/*right*). [53] (**c**, **d**) *In situ* "bottom-up" assembly of TMV-like nucleoprotein tubes as multivalent templates on solid supports. (**c**) Bidirectional growth from CP oligo- and multimers on immobilized RNA, starting at the RNA's origin of assembly (OAs) structure; (**d**) spatially selectively equipped, patterned Si substrate with nanotubes on meandered areas fashioned with RNA (*via* DNA anchors coupled to aldehyde groups). AFM topography image [54] (reproduced from [5, 53, 54] with permission)

systems: Proof-of-concept experiments *e.g*. with CPMV [10, 48] confirmed that dual-functionalized viral shells with at least one binding moiety to a target, and multiple signal-generating units (fluorescent dyes, which might be replaced by quantum dots or further detectable molecules in other formats) were able to significantly enhance the sensitivity of analyte detection. To achieve this effect, the virus-derived functional additives were applied as secondary reactants, attaching to a hapten located on the primary probe (*e.g*. by means of streptavidin presented on a highly fluorescent virus, which thereby targeted biotin labels of nucleic acid hybridization probes or antibodies; refer to Fig. 22.9).

22.8.3 Drug Delivery and Diagnostics by Help of Viral Nanocontainers

The above-described possibilities arising from the incorporation of two or even more functionalities into individual viral capsids have drawn special attention to their putative use in diagnostic and therapeutic medical applications. Most appealing fields of utilization are the targeted delivery of indicator and effector substances to specific cells or tissues inside living organisms. While specific biogenic target structures may be found and bound by molecules presented on the outer viral capsid, the load of the viral cavity might serve as an imaging agent or, alternatively, as a drug. Plant and many bacterial and animal viruses are regarded ideal for such purposes due to their capsid dimensions, their proteinaceous multivalent surface (as described before), and their expected biocompatibility: They do not have any known pathogenic potential for humans and are usually tolerated without any notable adverse effects, probably due to their regular uptake with all types of food, ranging from vegetables and fruit to meat as well as seafood.

However, viral nanoparticles including plant-derived ones were shown to elicit immune responses and to penetrate many cell types and organs. Therefore, prior to any medical application, the risk of strong immune reactions and toxic effects due to high doses or specific modifications of viral capsids needs to be minimized. This is why numerous lines of research and development currently focus on strategies shielding the viral protein surfaces by covalent coatings with inert polymers, mainly polyethylene glycol, which not only reduces immunogenicity but obviously also increases the elimination rates (clearance) of viral capsids introduced into the body.

Before use, suitable preparations of viral nanocontainers intended to act as delivery vehicles for imaging agents or drugs (or genetic information in the case of gene therapeutical approaches) have to be loaded. Viral cavities may be filled with the respective cargo by diffusion (Sect. 22.6.1), supported by accordingly charged groups exposed inside, or by *in vitro* assembly of the viral capsid in the presence of the substance of choice (encapsulation). Some virus shells undergo conformational alterations upon changes of pH or ionic environment, which can lead to a convenient opening and closing of pores thus facilitating loading and retention of certain molecules. Once taken up into target tissues of treated organisms, however, cargo release into the surrounding cellular or intercellular medium may be difficult to achieve in a controlled manner, which is why novel externally inducible nanocontainer opening or destruction mechanisms are matter of intense current investigation (see Fig. 22.10 for a proposed strategy to achieve drug release by alternating electromagnetic fields).

Finally, two completely different applications of viral templates deserve to be touched in this section, although without being subjects of comprehensive description. One application undergoes powerful novel developments since viral capsids have been introduced into medical research as engineerable nanoparticulate effectors: the vaccination of animals and humans. Here, especially plants have shown an interesting potential to produce immunogenic, but non-infectious epitopes of animal pathogens or

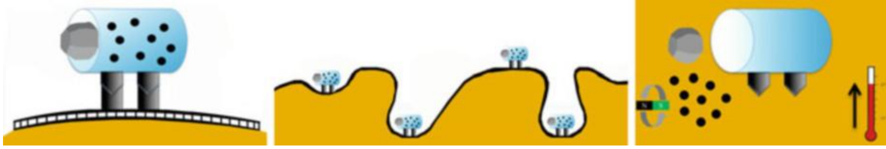

Fig. 22.10 Suggestion for a combination therapy against cancer, based on plant viruses. A plant virus is filled with a drug, which is thus shielded from blood and other cells. A modification with antibodies allows selective targeting of a tumor cell. The virus can enter the cell, and iron oxide nanoparticles (*large dots*) on or in the virus can be used for local hyperthermia, in combination with drug release (*small dots*). Hyperthermia requires an alternating magnetic field, applied externally. This and similar strategies are poised for breakthroughs in nanomedicine (image inspired by T. Pellegrino)

even tumor cells, either on the surface of chimeric plant virus particles, or as complete capsid structures in the case of heterologously assembled animal-viral particles. The resulting vaccines are devoid of any 'biorisk' of cell culture-derived contaminants. 'Edible' plant-derived mucosal vaccines against common diseases are under intense investigation, as well as plant viral particles equipped with peptide epitopes provoking immunogenic responses *e.g*. counteracting breast cancer [56]. The use of (engineered) VLPs and virions for vaccination has been described in detail in Chap. 21.

The last route of research presented here closes the circle back to the employment of viral templates in materials sciences: VLPs exposing peptides typical of extracellular matrix are used as novel functional coatings for tissue culture supports, thereby affecting adsorption and differentiation of animal and human cells growing on such substrates [57]. Those and related studies make use of the precise spacing of specific cell attachment signals if fused to viral CP subunits: By mimicking their spatial organization typical of certain organs, the viral hybrid particles are expected to help controlling the fate of interacting cells *in vitro*, with the aim of producing engineered tissue substitutes for a subsequent implantation into injured organs.

22.9 Other Systems

Mammalian viruses and their many uses for immunization and as gene vector could with full right be addressed as (rather advanced) nanotechnological instruments. This is however not usually done, due to their importance in medicine, and due to safety considerations, but probably also since most are more sensitive to environmental conditions than plant viruses (there are exceptions, though), and due to the small amounts available, compared with many plant viruses and phages, which can be obtained in grams. In any case, research and also applications in medicine are based on the infectivity for humans, but in nanotechnology this is always a disadvantage. However, enveloped viruses are used for arrays by exploiting their lipid layers.

A very good alternative are VLPs derived from animal viruses that can be prepared by removal of the RNA or DNA, or by assembly of pure CPs. Such nucleic acid-free virus shells are frequently employed for immunizations (see Chap. 21). For

the more complex animal virus particles (but not for simple viruses such as parvoviruses, nodaviruses and picornaviruses), assembly and surface chemistry may be technically more challenging than for simple plant viruses and phages. However, some specific properties of animal viruses and VLPs, such as their in-built capacity to recognize and penetrate specific cell types, including specific tumor cells, have made some of these virus particles (including engineered ones) relevant choices for the development of targeted gene therapy (since more than two decades), of drug delivery, and of other applications in biomedically-oriented nanotechnology. Details on the use of animal virus particles for biomedical and animal health applications are beyond the scope of this chapter, and the reader is referred to Chap. 21 regarding vaccine applications.

Archaea viruses attack archaea rather than bacteria, but many have a structure that is very similar to those of some phages. However, comparatively few are known, and their use in nanotechnology such as in combinatorial synthesis (phage display) is not established. The very high temperature stability of some archaeal viruses (especially hyper-thermophilic ones) may provide a stimulus for a broader use, though, and is interesting especially for the materials research side of nanotechnology.

Viroids are surprisingly simple infective agents, consisting only of an uncoated, tightly folded RNA molecule. Their discovery by Diener provided further stimulus in plant virology. In terms of physical properties and nanotechnology, they should be seen as a type of nucleic acid. While catalytic ribozyme sequences are very well known and used, viroids as a whole have not yet found applications in typical nanoscale science. With another view on nucleic acids, viral genomes feature many regulatory elements that have found use in heterologous protein production systems.

22.10 Perspectives and Conclusions

The challenges for virus nanotechnology are analogous to those for nanotechnology in general (Sect. 22.1). However, viruses imply the strict definition of nanoscale (< 100 nm), comparable to the smallest mass-produced structures. A major question is whether a (conventional) “top-down” or a bio-inspired “bottom-up” scheme is best suited. The answer has to be given for each structure separately; however, the smallest structures, of the size of viruses, might indeed require “bottom-up” and self-assembly strategies (Sect. 22.4). The use of viruses, especially for nonbiological nanotechnology (Sect. 22.1), requires “new surfaces”, chemically or genetically engineered, and tailored for size and function. The background knowledge developed for such chemical modifications (Sects. 22.2 and 22.3) can also be used for templated structures (Sect. 22.6), and it is key for most applications (Sects. 22.7 and 22.8). The latter range from very small demonstration devices up to true production schemes, where also questions of biosafety and waste management would have to be addressed. Although much work has been started, the whole process of virus modification to achieve the desired functionality and/or properties is far from being

solved, and especially on the molecular scale (<5 nm) trial-and-error approaches are still prevalent over completely rational engineering. Key to overcome this problem are new analysis and manipulation tools (Sect. 22.5). Viruses, due to their high geometrical and chemical definition and functional properties, will then have the chance to become more generally involved in future nanotechnological breakthroughs.

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Further Reading

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Also especially recommended for further reading are references [1–3, 7, 9, 24, 48, 51] *listed above.*