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Virus- and extracellular vesicle-inspired nanomedicine: lessons learned from nature

Master thesis

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Summary

Nanoparticles are nanosized compartments that can contain a diverse set of cargo. In the field of nano-medicine, much research is being performed on targeted drug-delivery systems, where nanoparticles are used for targeted delivery of therapeutics. Synthetic nanoparticles show promising results, however, many complications arise concerning biocompatibility, immunogenicity and toxicity among others. Biological nanoparticles, such as virus-inspired particles and particles influenced by extracellular vesicles, could provide us with new delivery systems that are mostly biologically compatible, and have a reduced toxicity. Moreover, the study of viruses and extracellular vesicles could provide us with more insights that could be incorporated into current biologically inspired nanosystems.

1 Introduction

1.1 *Nanotechnology*

Developments in the nanotechnology field have resulted in numerous applications ranging from solar cells to nano-foods. Even in medicine, nanotechnology has left its mark by creating new fields of research; nanodiagnostics and –therapeutics, leading to new concepts in diagnostics and treatment of disease. Targeted-drug delivery using nanoparticles is one of these concepts. Here, therapeutics are enclosed within a nanosized compartment, both naturally or synthetically based, and are targeted towards diseased tissue. The objective is site-specific release of the drug, which reduces detrimental effects to healthy tissue. The prospect of specifically targeted vesicles for the delivery of genes, drugs and other molecules to diseased tissue has spiked the interest in the research and development of inorganic, organic, biological or hybrid nanoparticles [1]. Targeted nanoparticles could enable early diagnosis and offer new concepts for treatment of diseases such as cancer. Currently investigated nanosystems are quantum dots (QDs), dendrimers, polymer vesicles, nanospheres, dextrans, liposomes, micelles and protein-based nanostructures such as viruses, and are often decorated with targeting moieties and filled with (cytotoxic) drugs and/or imaging agents [2, 3, 4]. Each of these systems has advantages and disadvantages in terms of biocompatibility, pharmacokinetics, toxicity and immunogenicity, however few of them have been well characterized in vivo [2, 3]. However, before this concept can be applicable, more research and understanding concerning the behavior of the different nanoparticle components is needed. Clearance rate, circulation time, distribution and accumulation, immune response, toxicity, penetration of the endothelial barrier, target cell attachment and entry, and endosomal escape are some of the processes that are involved in targeted drug-delivery, of which all need to be well understood and optimized before medical application can be achieved. All of these processes are not only influenced by the response of the organism, but also by the nanoparticle's characteristics; the size, shape, (chemical) composition, coating and the presence of ligands, all participate in the nanoparticle's behavior inside the organism. However, due to the influence of various characteristics, the system is tunable, meaning that all characteristics can be optimized in such a way that the benefits outweigh the negative aspects. The tunable ability of nanoparticles enables us to incorporate specific characteristics into the particles. Several biological nanoparticles have developed efficient ways of circulating through an organism and delivering their cargo in a site specific manner. If the characteristics that aid in the success of these particles could be identified and incorporated into either synthetically or naturally based particles, one might create an optimized particle that can efficiently reach its goal without causing detrimental effects. This would especially be helpful in the treatment of cancer, where current therapies are often accompanied with severe side-effects. A number of experimental studies show the feasibility of these systems as drug delivery platforms, but the only platform currently approved for clinical use is the targeted and drug loaded liposome system [2, 5, 6]. The fact that only one system has reached clinical use, shows that even though these systems are promising, more research on nanoparticle toxicity and biodistribution in vivo is necessary [3]. The biological behavior such as clearance from the body and toxicity of nanoparticles greatly depends on their size, shape, composition, surface chemistry and charge, and other associated physical properties [3]. One of the main issues concerning the use and applicability of nanoparticles is the potential toxicity and biocompatibility. Often, repeated administration of nanoparticles leads

to accelerated uptake and accumulation in the macrophages of the RES and thereby induces toxicity [7]. More understanding regarding nanoparticle circulation, clearance rates, blood half-life, stability, immunogenicity and organ biodistribution is needed for nano-scaled delivery vesicles to be as therapeutically or diagnostically useful as they can be [3]. Naturally occurring nanosystem such as viruses and exosomes could provide the field with new strategies that could aid in the formation of better systems. Virus- and exosome-inspired nanomedicines are highly investigated and several formulations have been constructed.

1.2 Nanoparticles based on biological systems

Virus-inspired nanoparticles

Viral nanoparticles (VNPs) are virus-based nanoparticle constructions [2]. They can be bacteriophages, plant or animal viruses, and infectious or non-infectious. Virus-like particles (VLPs) are a subset of VNPs that lack genomic content, making them non-infectious. VNPs are dynamic, self-assembling particles that form highly symmetrical systems, polyvalent and monodisperse structures. They are robust, modifiable and can be produced in large quantities within a short period of time. The main advantages VNPs have over synthetic nanomaterials are that they are biocompatible and biodegradable, as they are primarily protein-based [2, 4]. VNPs derived from plant viruses and bacteriophages are particularly advantageous as they do not cause human disease [2, 4]. Due to their nature, plant-based VNPs are less likely to be pathogenic in humans and therefore are less likely to induce undesirable effects. A variety of different VNPs is available, and each kind can be tailored for a specific application as they are not ready-made nanoparticles (figure 1). Viruses are relatively rigid structures, thereby enabling the display of different moieties on the surface of the VNP [1, 4]. Via conjugation chemistry, and/or genetic engineering, different functions can be provided to a specific VNP [2, 1]. Small chemical modifiers, peptides, proteins and even additional nanoparticles can be attached to VNPs via genetic engineering, chemical bioconjugation, mineralization, or encapsulation techniques [2]. They have been used and developed for a variety of applications including, fundamental structure-function studies of viruses, phage display techniques for the selection of peptides and proteins; new vaccines; delivery of therapeutic genes into specific cells (gene therapy) and targeted delivery of drugs using viral particles as nanocarriers or nanocontainers [2, 1]. Spherical VLP's that are currently under investigation for these applications include Cowpea chlorotic mottle virus (CCMV), Cowpea mosaic virus (CPMV), bacteriophages and polyomavirus [3, 4]. And even though VNPs provide a robust and stable platform, they are also dynamic in their structure and many of them undergo transitions in their conformational structure, leading to the formation of pores [2]. These and other characteristics make VNP's suitable candidates for targeted drug-delivery.

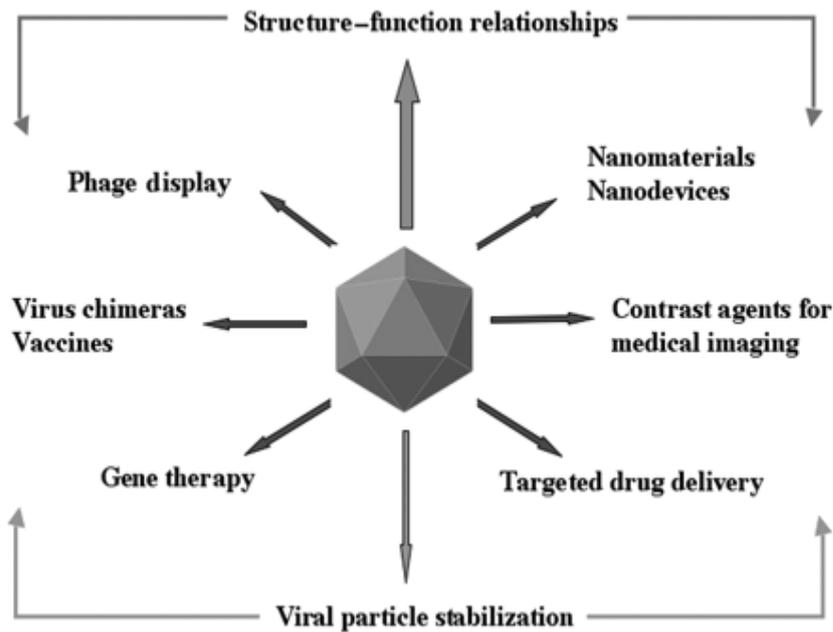


Figure 1: Applications of VLP's in the biotechnology and nanotechnology field. Studies on structure-function relationships of viruses are used for the development of new applications. Viral particle stabilization through protein engineering are important for these applications. Adapted from [1]

Extracellular vesicle-inspired nanoparticles

The application of exosomes as nanocarriers is accompanied by numerous benefits; they are naturally occurring biological products and therefore induce a low to absent immunogenic response. They are relatively easy to handle, and due to their metabolic inactive state, they are very stable and can be stored for over 2 years while retaining their biological activities [8]. It has been found that exosomes are able to deliver siRNA and tumor-suppressive miRNA to cancer cells, which led to the suppression cancer cell growth [9]. Exosome-based vaccines also show great potential. They generate and potentiate an immune response against a diverse set of tumours [8]. Exosomes derived from dendritic cell cultures, loaded with tumour antigenic peptides were used as vaccines and were administered to metastatic melanoma patients. There was no sign of toxicity and an increase of NK cell numbers with restored NKG2D (an activating NK cell receptor sensitive to genetic stress among others) expression. Moreover, the antigen-presenting ability of exosomes is twice as long as that of APC cells. However, there are some restrictions. So far, exosomes cannot be synthesized. They have to be extracted directly from body fluids or cell culture media. More so, all experiments and therapies rely on exosomes derived from the same patient they are used on. And during the extraction process, the number of exosomes can vary, which can be problematic in establishing an adequate dose [8]. Thus, exosome-based nanotechnology is promising, however, new techniques for the production or extraction of exosomes are needed; finding compatible groups or developing synthetic exosomes could provide a resolution for this problem. Moreover, synthetic exosomes could provide a solution for the extraction process and possibly create new applications, such as drug delivery vesicles of non-native therapeutics.

This thesis discusses the characteristics of viruses and exosomes that aid in their success as natural nanoparticles, the progress made by incorporating certain factors into virus- and EV-inspired nanosystems and discuss the challenges that still remain concerning biological nanoparticle lifetime and efficiency.

2 Viral characteristics enable viral success

Viruses are small, infectious nanoparticles that require living organisms for self-replication. They are natural nanoparticles that have developed an elegant way of sustaining their existence. By infecting specific cells, they can either induce replication and disseminate to other areas, or remain in a latent phase. Viral success relies mostly on their ability to self-replicate and produce an enormous amount of offspring. Their numbers ensure their success, and sustain the cycle of infection and replication. During this repetitive cycle, viruses have evolved into structures that have developed a coordinated assembly and disassembly process and are able to deliver their content in a site-specific manner [10]. Due to years of research many viruses are well characterized, however, the viruses that are investigated are small fractions of the total predicted virus diversity present in the biosphere. It is even suggested that viruses are the most abundant biological entities on the planet [11]. Even with all of this diversity, most viruses share several general characteristics. They consist of repeating subunits that assemble into highly symmetrical and homogenous structures that enclose their genetic material [12]. Some of these subunits are able to self-assemble into non-infectious particles, devoid of genetic material. Other viruses require genetic material to form functional particles. The virus particles can vary in size and shape, ranging from 18-500nm in diameter for icosahedral structures and >2um in length for several filamentous or rod-shaped viruses (also see figure 2) [10]. The symmetrically organized particles are usually not static; many viral capsids show pleomorphism, the ability to assemble into a range of different architectures, which enables adaptation to the environment [13]. With surface-exposed ligands, viruses navigate within their host, targeting specific cells and avoiding the hosts defence mechanism [10].

2.1 Evasion of the innate immune response

The ability of viruses to survive and avoid degradation by the host's immune system is a result of millions of years of evolution [14]. Viruses use their (non-) structural proteins to modulate or interfere with the immune response to avoid clearance. For pathogens, the innate immune system is the first line of defence they encounter, and roughly consists of the innate immune cells (monocytes/macrophages, dendritic cells (DC), natural killer (NK) cells, and NK-T cells), the complement system and the endothelial barrier. All facets of the innate immune response work together and try to eliminate the foreign substance. Viruses are equipped with several strategies that enable them to evade the immune response, however, these strategies are not always successful. Nonetheless, much can be learned from these strategies. It appears that many of these survival mechanisms are regulated by structural characteristics. Virus size, shape and surface charge, which are determined by viral proteins, can aid in evasion of the immune system.

Size and shape

All foreign particles that enter the blood stream are eventually taken up by phagocytic cells. However, for efficient phagocytosis to occur, these particles need to be larger than 500 nm, and preferably between 1 and 3 um. Studies have found that range is optimal for efficient phagocytosis [15, 16, 17]. Particles smaller than 500 nm will be less efficiently taken up [18, 17]. Coincidentally, the size of icosahedral viruses ranges from 18-500 nm, which makes viruses less susceptible to

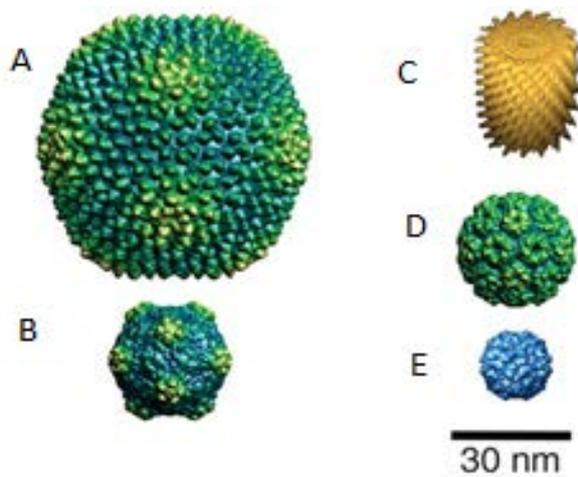


Figure 2: Cryo-electron micrograph and image reconstructions of icosahedral and helical viruses. A, murine polyoma virus, 51nm diameter. B, cowpea mosaic virus, 31nm diameter. C, the rod-shaped TMV, which measures 18 by 300 nm. D, CCMV, 28nm diameter. E, satellite tobacco mosaic virus, 18-nm diameter. Adapted from [10].

phagocytosis by their size alone [10]. Non-icosahedral viruses, including rod- and elliptically shaped viruses, have another benefit besides small size. The shape of a viral particle has also been shown to influence phagocytic uptake. Particles with a spherical shape are more easily taken up, than particles with a more elongated shape, such as rods or elliptically shaped particles [15, 19]. Viruses with these divergent shapes such as the tobacco mosaic virus (TMV), a rod-shaped plant virus with a diameter of 18nm and a modal length of 300nm, has been shown to circulate in the bloodstream for a longer period of time and appears to be less rapidly cleared from tissue than its icosahedral counterparts [20, 15, 22, 23, 24, 25]. However, TMV does show a fast blood clearance rate, a characteristic that might be favorable in nanoparticles, as particles that are not cleared

in an adequate timeframe could become toxic. However, most viruses are icosahedral, and thus cannot benefit from this particular characteristic. There are viruses that are more flexible in their appearance. These viruses, with hepatitis C virus (HCV) as example, are pleomorphic; they contain a flexible envelope that surrounds their capsid and enables them to (slightly) adjust their shape. And as it has been shown that phagocytic cells have a preference for rigid particles, a characteristic most likely due to a mechanosensing mechanism, it might be that flexible viruses have an advantage over the more rigid viruses [17]. However, direct evidence for this possible effect remains to be found.

Surface chemistry

Another factor contributing to the efficiency of phagocytosis is a particle's surface chemistry, and in particular its surface charge. It has been shown that hydrophobic particles are more susceptible to internalization than hydrophilic, non-ionic particles. This is due to the immediate opsonization that hydrophobic particles evoke [26]. The impact of surface charge on phagocytosis has also been described in [4], where they show that positively charged particles have higher circulatory times than negatively charged particles, indicating a decrease of clearance by phagocytosis. Positively charged particles are less efficiently opsonized and are therefore able to circulate for a longer period of time. The surface charge of viruses greatly depends on their environment. They have a pH dependent surface charge, where the isoelectric point (pI), the pH value where the net surface charge switches from negative to positive, determines whether the particle would portray a positive charge or a negative charge when present in polar medium [27]. [28] demonstrated this by measuring virus adsorption to charged particles under different pH circumstances. Viruses with a pI lower than the pH of the medium showed a negative net surface charge (viral pI < solution pH = negative surface charge), while viruses with a pI higher than the pH of the medium showed a positive net surface charge (viral pI > solution pH = positive surface charge). And as expected, viruses (poliovirus 1 strain Brunhilde; pI 7.1) with a pI similar to the pH of the medium, showed affinity for both negative and

positive particles as isoelectric particles are likely to neither repel nor attract charged surfaces. When placing this in the biological context of phagocytosis, viruses with a low isoelectric point (in pH 7.4) will be more likely phagocytized than viruses with a high isoelectric point, as their surface charge will be negative, thereby favoring phagocytosis. This is most likely due to the increased affinity of opsonins for negatively charged surfaces [7, 29]. Viral pI's lie in the range between 2.1 and 8.3 with a mean of approx. 5.0 [27]. This suggests that the majority of viruses have a negative surface charge when present in human fluids such as blood plasma, which would favor phagocytosis. Only a few mammalian viruses including mengovirus, poliovirus, and rotavirus have pI's higher than 7.4.

Viral surface proteins

As discussed above, the combination of viral size, shape and surface chemistry is involved in viral immune escape. However, it appears that efficient immune evasion is not dependent on these characteristics; they only seem to aid in the process. The complex and numerous mechanisms viruses have to evade the immune system (reviewed in [30]) mostly include viral proteins either structural or non-structural. Evasion mechanisms regulated by viral proteins include interference with antigen presentation by MHC-I receptors, evasion of NK cell responses, evasion from CTLs by antigenic variation, evasion through latency and mimicry. These are only a few examples of strategies that viruses use. Some viruses have the ability to prevent interaction of MHC-II molecules with the T-cell receptor (TCR). The transmembrane glycoprotein gp42 of the Epstein-Barr virus (EBV) interacts with MHC-II molecules at various stages of the virus' maturation, where it hinders antigen presentation to CD4+ T-cells. The envelope protein of HIV-1, gp120, binds to CD4 molecules and similarly inhibits CD4 and MHC-II interaction. The HCV major envelope protein E2 binds to CD81, a tetraspanin present on the surface of T, B and NK-cells. The interaction prevents NK cell mediated lysis and cytokine release. Some viruses, mainly RNA viruses, display high antigenic variation. The viral polymerase are prone to errors and combined with the high replication rate, they make variable structural and non-structural proteins, thereby generating distinct viruses or 'quasi-species'. Under selective pressure from CD4+ cells and neutralizing antibodies for a specific virus, the virus with the 'escape' mutation will not be recognized and will accumulate in the host. When an anti-viral immune response against the 'escape' mutant is generated, another 'escape' mutant will rise upon which a new cycle begins. HIV-1, HCV, herpes viruses and others, use this strategy for escape. Herpes viruses have developed an additional strategy which they use during their latent phase. They reduce the number of epitopes they expose on their surface and thus reduce the number of 'red flags' the immune system can recognize [31, 32, 33]. Human Papilloma virus (HPV) modulates, among others, the release of macrophage chemoattractants, via its viral proteins, thereby reducing the influx of macrophages to the site of infection [34, 35]. Others, such as HCMV and HCV, use the infection of immune cells as a strategy against the antiviral immune response. They infect macrophages and use them as means of interference with the immune response and as vessels for further dissemination [36, 37, 38].

Some viruses are equipped with a shield, an envelope layer consisting of highly glycosylated carbohydrates that form a barrier and reduce recognition by the immune system. Such form of protection is seen in vaccinia virus, HIV-1 and its simian counterpart SIV-1 [39, 40]. The viral envelope is acquired upon release of virions. It contains mostly lipids and proteins derived from the host. Mature vaccinia viruses that are surrounded by the envelope bilayer are resistant to the complement system due to the presence of host complement control proteins (CD55 and CD59) in the envelope [40]. A similar strategy is observed in HIV-1 [41]. The envelope proteins of HIV-1, gp120 and gp41, are also found to bind to factor H, a protein that is involved in the inhibition of complement-mediated

destruction. Acquiring an envelope appears to be a good strategy for immune evasion; host proteins aid in complement inhibition and are in some cases used as means of infectivity.

Plant viruses

Animal viruses are very capable of modulating and evading our immune system as they have co-evolved together with their mammalian hosts. However, plant viruses did not have this advantage. As they have a different type of host, they most likely developed other mechanisms of infection than needed for infection of mammals. This notion is strengthened by the assumption that plant viruses are not able to infect or replicate in mammalian cells. Due to the lack of infective or replicative abilities in mammals, many plant viruses are being investigated as possible drug delivery systems. They can be easily altered and the chances of unwanted infections are considered to be low. However, as these viruses are plant viruses, not much is known about their behavior in mammals, therefore research on how they behave in a mammalian environment is important to perform. Recent research has shown that some plant viruses such as TMV are able to evoke an antiviral immune response in mammals, which contradicts previous statement concerning their safety [42].

2.2 Crossing the barrier

Two main surfaces are important concerning virus entry; the skin and the mucosal epithelium [43]. Both are lined with specialized epithelial cells that provide a tightly regulated boundary. Some viruses have developed methods of circumventing these boundaries by making use of vehicles such as mosquitos that penetrate the skin and create a direct entry pathway into the bloodstream. However, even when the first barrier is evaded, the inner lining of the vasculature provides a second barrier; the endothelial barrier. This barrier lines the walls of the vasculature and lymphatic vessels [44]. It is a tightly regulated border, where transport of (macro) molecules, cells and liquids are controlled, and crossing the barrier usually requires some effort. The mechanisms behind penetration of the barrier are mostly shared by both enveloped and non-enveloped viruses, and do not depend on the presence or absence of an envelope. Penetration of the epithelial barrier occurs mainly via three mechanisms that are also observed in the penetration of the endothelial barrier: endocytosis and transcytosis of the virus without infection, polarized surface entry and infection by fusion and endocytosis of the virus with endosomal fusion leading to infection [43]. However, we will only discuss the entry mechanism where no infection of the epithelial cells is initiated, as this is a characteristic aspired for nanoparticles.

Transcytosis

Transcytosis is a transport mechanism that is usually reserved for the transfer of large molecules such as albumin across the barrier without disrupting its integrity [45]. The transport of albumin mainly occurs via fission and budding of caveolin-1 (Cav-1) rich microdomains present on the luminal side of the cell, resulting in caveolar vesicles that transport the cargo to the basal side of the cell, where they fuse and release content. To initiate transcytosis, interaction with the appropriate receptor is required. For albumin this is gp60 [46]. Interaction results in receptor clustering and subsequent interaction with Cav-1, which eventually leads to fission. Viruses use transcytosis as a means of entering the extravascular space as it does not involve infection of the transporting cell [45, 43]. Both enveloped and non-enveloped viruses have been found to use this mechanism. HIV-1 is believed to use transcytosis during the first steps of primary infection [47]. Its viral glycoprotein,

gp120, interacts with its alternate receptor galactosyl ceramide, a sphingolipid that is thought to form microdomains on the apical side of epithelial and colon cells, which results in receptor-mediated transcytosis [47, 48]. The HIV-1 glycoprotein gp120 is also been found to interact with mannose-6-phosphate receptor. This interaction induces a different type of transcytosis; adsorptive transcytosis (transcytosis that is triggered by electrostatic interactions between cationic viral proteins and anionic microdomains of the plasma membrane of brain capillary cells), which enables free virus to cross the blood-brain barrier [49, 50]. Other viruses use more complex strategies. The Epstein Barr virus (EBV) interacts with preexisting IgA antibodies, that are specific for the surface protein gp350, and forms a complex that interacts with the poly-immunoglobulin receptor present on the basal surface of epithelial cells [51]. This leads to transcytosis of an infectious particle across the epithelial barrier. However, when the formed complex interacts with another cell type, infection would be initiated. Viruses can also be transcytosed via M-cells; specialized epithelial cells that lie in immune-associated patches (Peyer's patches) [43]. It is thought that these cells transport poliovirus, HIV-1 and reovirus across [45]. Transcytosis is also used in penetration of the endothelial barrier. Certain strains of the West Nile virus (WNV) cross the endothelial barrier via transcytosis, which depends on interactions between the viral envelope protein of WNV and host cell receptors [52].

Paracellular transport

Some viruses such as HCV can cross the barrier via a passive manner. Several areas in the vasculature portray an intrinsic high permeability, due to the presence of fenestrae and sinusoids. These types of vessels are mainly found in specific organs, such as endocrine glands, kidney and liver, where fast exchange of nutrients, molecules, fluids and other components is needed [53]. Tumor vasculature also possess these characteristics, however, tumor vasculature permeability is a result of unintentional architectural flaws, unlike the pores of kidney and liver sinusoids [54]. These pores lack a diaphragm and basal lamina, which makes them extremely leaky [53]. They are in general 150-175 nm in diameter and occur quite frequently (9-13 per μm^3) [55]. HCV exploits this characteristic and extravasates quite easily due to its small size, into the extracellular space, where it infects its target cells. HCV also contains a flexible lipid envelope that is thought to aid in the extravasation process as well. In areas that do not contain fenestrae, paracellular transport is only reserved for particles $<3\text{nm}$ [45]. However, several viruses have developed mechanisms to increase the endothelial permeability. The gp120 viral protein present on HIV-1 is also able to induce disruption of tight junctions in the mucosal barrier, and thereby increasing local permeability to the virus. Interestingly, mere exposure of HIV-1s gp120 is enough to increase permeability [56].

2.3 Target cell attachment and entry

The first step in viral infection is attachment. Without target cell attachment, subsequent events such as internalization and genomic delivery would not occur. By interacting with specific membrane proteins and/or receptors that facilitate virus entry, viruses can induce conformational changes in either viral entry proteins or host receptor proteins, leading to internalization of the virus particle [57]. Both enveloped and non-enveloped viruses need to attach to target cells prior to internalization. The attachment factors and entry receptors are predominantly virus specific, therefore this section is restricted to the similarities found in viral protein structure, interactions and entry mechanisms.

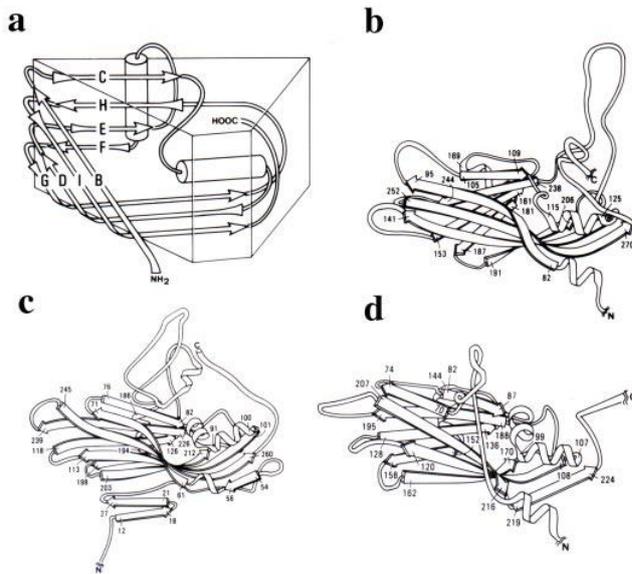


Figure 3: Structural representation of the viral capsid proteins of poliovirus. 60 copies of viral proteins VP1, VP2, VP3 and VP4 form the icosahedral virion. VP1, -2 and -3 share a common morphology. A, a representation of the eight-stranded beta-sandwich that is shared by VP1, VP2, and VP3 and the capsid proteins of a number of other icosahedral viruses. B-D, Ribbon diagrams of VP1, VP2, and VP3 displaying the shared beta-sandwich structure with the unique loops and terminal extensions of each of the subunits. The N-terminal extensions of VP1 and VP3 are not shown. Adapted from [59].

Viral protein structure

The receptor-recognizing viral proteins are often embedded in the viral capsid or envelope, where the receptor-recognizing surfaces are displayed in the form of spikes (present in coronaviruses and AAV's) or canyons (present in picornaviruses and polioviruses) [58]. Non-enveloped viruses have been characterized quite well, due to their small size and high stability. The structure of enveloped viruses on the other hand, is more difficult to decipher as they do not crystallize well. Only a few examples, including SFV and dengue virus, have been fully characterized. Viral entry proteins are usually glycosylated oligomers [58]. In some viruses such as alphaviruses, the entry proteins form heterodimers with other viral proteins and interact with each other to form an interacting lattice. The cooperation of viral proteins is also seen in many non-enveloped viruses, where the entire capsid is formed by proteins involved in entry. Other viruses, including adenoviruses and retroviruses, contain

entry proteins that do not show this interaction pattern, and exhibit a more independent mechanism of action. Interestingly, a similarity is observed between the topology of different viral entry proteins. For example, the spike structure, consisting of a stem and a globular head, is shared by a variety of different viruses including adenoviruses and reoviruses. Interestingly, the amino acid sequences that form the spikes do not share any similarity. More parallels are found between envelope proteins of influenza (HA) and respiratory syncytial virus (RSV) (fusion protein). It seems that even though the amino acid sequences differ, the structure of viral entry proteins remains similar. This is especially noticeable for non-enveloped proteins (derived from mammalian, plant or insect viruses) that seem to share the same basic structure, consisting of an 8-stranded β -barrel with a jelly-roll motif (figure 3) [59]. Unlike the common eight-stranded β -barrel, the binding pocket including its location and structure can differ between viruses. Some are located in a deep canyon, or contain loops, cavities and channels. There is also no connection found between the structure of the entry protein and the structure of the receptor; entry proteins can bind to a range of receptors, as seen in retroviruses, and a single receptor, such as sialic acid, can serve as an entry receptor for many viruses [58].

Viral protein-host receptor interactions

There are a variety of different virus receptors known and described [60], and despite the obvious differences, the majority of receptors are involved in cell adhesion and recognition [58]. There are also some general factors that contribute to viral attachment. Factors such as sialic acid and heparan sulfate (HS) are considered to be general attachment receptors. They are widely distributed and are

found in the extracellular matrix and on the surface of cells [126, 127]. Heparan sulfate carbohydrates presented on proteoglycans (HSPG) form interaction sites for at least 16 mammalian viruses, including HPV, HCV and HSV [128, 126, 127]. These interactions depend on hydrogen bonds, where the highly negative charged or polar residues of HSPG interact with positively charged viral proteins [126, 127]. SA is found on all eukaryotic cell surfaces, capping different oligosaccharides present on glycoproteins and –lipids [129, 134]. Several viruses, including polyomavirus, influenza virus (A, B and C), Coxsackievirus A24 and Enterovirus 70, use SA as an attachment receptor [reviewed in 134]. Other viruses such as certain strains of the polyomavirus and influenza virus use SA as a primary entry receptor [129, 130].

The interactions between viral proteins and host receptors are usually reversible and multivalent (avidity-determined), and viruses can use different interaction patterns to achieve attachment and internalization. The interaction between the viral entry protein and cellular receptors can be of high affinity, an effect that is often observed with receptors that usually show a low affinity towards their natural ligand [131]. Some viruses interact with receptors that show a combination of these characteristics; they bind to multiple receptors with high affinity. How strong the interaction between the viral entry proteins and their receptors is, is an important aspect in the internalization process. Typically, low-affinity interactions do not induce conformational changes as seen with the interaction of influenza's HA with sialic acid. High-affinity interactions, such as those observed between HIV-1's gp120 and CD4, do induce large conformational changes. However, the high-affinity binding of the adenovirus fiber to CAR is an exception, which does not induce any change in either protein (entry or receptor). Conformational changes, either in the viral protein or in the cellular receptor, are sometimes required for entry [58]. They can reveal additional attachment sites that enable binding of co-receptors. HIV-1's gp120 interacts with CD4, which induces a conformational change and leads to the binding of either CXCR4 or CCR5. In turn, this interaction results in the exposure of fusogenic sequences that lead to internalization. On the other hand, low-affinity interactions can also initiate entry, as seen for interactions with heparin sulfate. However, the precise role of many entry receptors is not known.

In absence of a native entry receptor, some viruses use alternative receptors or methods, of which little is known. Some evidence suggests the use of lipid rafts. These are lipid domains present in the plasma membrane that are enriched in cholesterol, glycosphingolipids and glycosylphosphatidylinositol-anchored proteins, and have been found to be utilized by viruses, including SV40, echovirus 1 (EV1), HIV-1, measles virus, Ebola virus and Epstein–Barr virus (EBV), for entry [58]. Several other viruses, such as the enveloped influenza virus, use lipid rafts to concentrate enough viral molecules (HA) to form effective virions [132]. However, the involvement of lipid rafts in the entry process remains controversial.

Viral entry mechanisms

Viral attachment to a target cell leads to the induction of either an endocytic or a non-endocytic internalization route [58]. There are several endocytic routes that viruses can exploit; clathrin- or caveolae-dependent endocytosis, and clathrin- or caveolae-independent endocytosis [57]. Which endocytic route is taken, depends on the entry receptor and/or the size of the virus. Small viruses, such as Simian virus 40 (SV40) are endocytosed via a clathrin- or caveolae-mediated endocytosis, which form vesicles of 100nm in diameter [18, 133]. Larger viruses can either be taken up via phagocytosis (immune cells), and via macropinocytosis, a process where vesicles of 0.2-10 um in

diameter are formed [18]. The non-endocytic route involves direct transfer across the plasma membrane. Viruses such as herpes viruses and retroviruses seem to enter cells by direct fusion with the lipid bilayer [60]. However, it has been found that the fusion of HIV-1 is preceded by receptor-mediated internalization [61], and there have been reports of HIV-1 using both endocytic as well as non-endocytic routes [58].

The efficiency of entry greatly varies between viruses. The adeno-associated virus serotype 2 (AAV-2), SFV and influenza can cross the membrane within seconds and have an entry efficiency of over 50%, while HIV-1 needs >1 minute before to enter its target cell. Moreover, the efficiency of this entry is only 0.1%, compared to AAV-2 [58]. The efficiency and kinetics of viral entry are most likely related to the viral morphology. Viruses that use low pH as entry trigger and have flattened structures such as SFV, seem to have optimal kinetics and entry efficiency.

Plant viruses are believed not to enter mammalian cells, as they are not able to use mammalian receptors for entry. However, recent evidence shows that the plant virus CPMV is able to infect mammalian cells expressing a specific protein, vimentin, which is expressed on the surface of certain cells [62]. Vimentin is a cytoskeletal protein that is expressed on the surface of fibroblasts, dendritic cells, endothelial cells, macrophages and lymphocytes. Interestingly, vimentin is overexpressed in many solid tumors, which might explain the nature of CPMV to accumulate in tumor tissue [63]. The viral ligand for vimentin is not known, but research shows a dependence on the presence of cholesterol.

3 Extracellular vesicles and how they function

Extracellular vesicles (EV's) are small, membrane-enclosed compartments that are released by almost every cell type found in the human body [64]. Their presence was observed in the 1980's, and continued research has resulted in an expansion of information about these naturally occurring nanoparticles. These vesicles share some similarity with retroviruses and some even propose that they share a common ancestor [65].

Based on their size and origin, two distinct groups of extracellular vesicles have been formulated; microvesicles (MVs) and exosomes [8]. Microvesicles are derived from the plasma membrane and have a size ranging between 100nm – 1µm. Exosomes are generally smaller and have a size < 100nm [8]. They are formed in the cytoplasm and released after fusion of multivesicular bodies with the plasma membrane. Extracellular vesicles share a resemblance with retroviruses, which makes them very interesting in the context of nanomedicine as both are seen as natural nanoparticles [8]. Retroviruses and exosomes have been found to share certain aspects. They contain a lipid bilayer, share a common glycan coat, and are even enriched in similar proteins and genetic material. Therefore, it has been hypothesized that they use similar biogenesis and entry routes. Moreover, exosomes have been found to contain viral miRNA and contribute to infectivity and communication of retroviruses.

All exosomes are believed to be involved in cell-to-cell communication, either supporting or disturbing (when originating from cancer cells) biological processes [8]. Which processes they influence depends mainly on their origin, but they have been implicated in facilitating and repressing the immune response, apoptosis, angiogenesis, inflammation and coagulation. Also reported was that EV's could act as morphogen transporters that determine polarity during the development and differentiation, and that they aid in the removal of unnecessary or deleterious molecules from cells without a secretory mechanism [8]. Exosomes can modulate cells via several mechanisms; interaction via ligand-receptor interaction, receptor transfer via membrane fusion, and altering cell activity via horizontal transfer of proteins and genetic material [66]. EVs can contain very diverse cargo; surface-bound receptors and ligands, genetic material, virus particles, proteins, prions and even organelles have been found in extracellular vesicles [67]. Some of these proteins are embedded in the lipid bilayer that surrounds the exosome. The composition of the bilayer resembles the parental plasma membrane and contains lipids, tetraspanins, adhesion molecules, and other membrane proteins. However, some lipids and proteins are enriched in the EV bilayer, while others are depleted. This results in a similar, but distinct surface.

Extracellular vesicles during cancer

Due to their involvement in cell-to-cell communication, EV's have been implicated in the spread of oncogenes [8]. It has been shown that cancer cells secrete exosomes and that these aid in the cancer progression and metastasis [9]. Microvesicles containing the truncated and oncogenic form of the epidermal growth factor EGFRvIII, which is often expressed by aggressive human brain tumours, are found to transfer this receptor between healthy glioma cells. The EGFRvIII containing microvesicles are released into cells surroundings and can merge with cancer cells that do not contain the truncated growth receptor. This leads to altered activity of the cell, where the oncogenic receptor activates and transforms signalling pathways and consequently changes the expression of EGFRvIII regulated genes. Tumor-derived exosomes containing oncogenic miRNA are shown to be excreted

during cancer invasion and metastasis [9]. Furthermore, exosomes have shown to promote survival of cancer cells by eliminating introduced drugs from cells, enabling resistance against these drugs [8].

3.1 *Evasion of the innate immune response*

Circulating EV's have been found in a variety of bodily fluids, including blood, urine, saliva, and cerebrospinal fluid among others [68]. Although the exact tissue origin is difficult to determine, it is generally believed that they are derived from mixture of many different cell types [69], indicating a relatively long circulation time. How exosomes are degraded is not known, however, it is likely that the process of opsonization is involved, as this mechanism is implicated in the degradation of other membranous vesicles [70].

EV's contain several proteins that aid them in evading degradation. Human APC-derived exosomes contain the glycosylphosphatidylinositol (GPI)-linked complement regulatory proteins CD59 and CD55. CD59 inhibits the formation of the membrane attack complex and CD55 inhibits the deposition of C3b, a complement factor involved in opsonisation and the formation of the membrane complex [70, 71]. The presence of these proteins enables resistance of the exosomes to complement regulated lysis. More evidence indicates that this is a general mechanism for exosomes to evade degradation by the complement system.

The various lipids present in the bilayer of exosomal vesicles have also been implicated in processes that lead to the evasion of immune clearance. Sphingomyelin and cholesterol are frequently found in the exosomal bilayer. These lipids are believed to be responsible for the tight packing and low water permeability of the exosomal vesicle. By forming hydrogen bonds within the bilayer, these two lipids are thought to increase the rigidity and stability of the vesicle, and protect it against detergents [66, 72]. GM3 has been implicated as a stabilizer of the exosome bilayer and is thought to shield the vesicle from interactions with blood components. Together with sphingomyelin and cholesterol, GM3 was shown to decrease uptake by the reticuloendothelial system (in vitro and in vivo). However, this effect was only found with low concentrations of GM3 in the membrane layer [66]. And the majority of exosomal vesicles eventually do end up in phagocytic cells.

3.2 *Crossing the barrier and target cell attachment*

Transport of extracellular vesicles across the epithelial barrier, is a process of which not much is known. The majority of them extravasate in a passive manner, where they exploit the presence of discontinuous areas in the epithelium, as observed in the sinusoids of the liver, inflamed tissue and tumor vasculature (discussed previously). The event occurs similar to that seen in exogenous nanoparticles of similar size, where particles smaller than 150nm can pass through the fenestrae of the liver. In tumor vasculature, particles as large as 500nm can pass readily through [91]. And due to the enhanced permeation and retention (EPR) effect present in solid tumor, extravasation into tumor tissue might be even favored.

It has become clear that all components that make up an EV are involved in its function. Lipids, membrane proteins and EV cargo are all involved. In the process of entry, the major contributors are

the membrane proteins. However the presence of specific lipids is important for exosomal function and therefore is described here as well.

Proteins

The membrane proteins found on EV's also have a diverse set of functions. They can trigger cellular responses, (prevent) interactions with the extracellular matrix, induce binding and fusion with target cells, and are involved in the assembly of exosomes [66]. Similar to lipids, some proteins are found commonly in the surface of EV's, while others are cell-type specific [73]. Commonly found membrane proteins include MHC-1, several cell adhesion molecules (lactadherin, thrombospondin-1, integrins, claudin-1 and ICAM's), signalling proteins (such as 14-3-3 proteins, GTPase HRas, Syntenin-1 and more), tetraspanins (CD9, CD63, CD81 and CD82 antigen) and several trafficking and membrane fusion proteins (such as annexins and syntaxin-3) [66, 73].

EV's derived from dendritic cells are found to contain MHC class I and II and a wide range of tetraspanins in their membrane. They are known to bind to certain integrins and MHC molecules, but other ligands have not been found [74]. Tetraspanins are often enriched in exosomes. Which tetraspanin(s) is (are) enriched, depends on the parent cell. Tetraspanins are involved in fusion, cell migration, cell-cell adhesion, and signalling. The most commonly found and enriched tetraspanins (CD9, -63, -81 and -82) have been implicated in facilitating the interaction between EV's and target cells [66]. The tetraspanins CD9 and CD81 have been shown to mediate attachment/fusion of DC-derived exosomes with BMDC target cells [75]. These tetraspanins have shown to induce fusion in other cell types as well. However, this function has been contradicted by studies performed with mononuclear phagocytes [76]. Here, CD9 and -81 knockout alveolar macrophages formed 3 to 4 times more multinucleated giant cells than control cells, an effect that is not expected if these tetraspanins induce fusion. It appears that another tetraspanin, CD63, is responsible for fusion events in these types of cells. This indicates that the function of tetraspanins depends on the cell type.

Adhesion molecules present in the lipid bilayer include integrins, thrombospondins and ICAM's. The most abundant of these adhesion molecules are integrins. It is believed that integrins are involved in addressing the exosomal vesicles to specific target cells. Integrins form heterodimers that are comprised of two subunits (α and β). There are 24 different integrin heterodimers identified in humans, which primarily function as adhesion molecules for cell and extracellular matrix binding. Depending on the parent cell, exosomes display specific integrins on their membrane. As every integrin heterodimer has a different ligand, the preference for a specific cell of a certain exosome will be mostly dominated by the specific set of integrins present in the bilayer. The affinity of interaction between the integrins and their ligands is also affected by cell type. The composition of the membrane is believed to have influence on the interaction between integrins and their ligands. A difference of affinity has been observed between parental plasma membranes and exosomal membranes, most likely due to the different membrane compositions. The exosomal membrane contains certain enriched components that the parental membrane does not have, which might modulate the interaction between integrins and their ligands [66]. Thrombospondin-1 and-2 have been found on exosomes derived from various types of cancers [66]. Thrombospondin-1 contains a RGD-domain that binds to $\alpha v \beta 3$ integrins and knockdown of Thrombospondin-1 in cancer cells reduced their migration and adhesion in vivo [77]. Thrombospondin-1 might have a similar function in exosomes. Several types of ICAM's (1 and 3) are also found in certain types of exosomes. They are believed to be involved in the mediation of immune response. ICAM-1 is a ligand for integrin $\alpha L \beta 2$

(LFA-1) and Mac-1, and promotes leukocyte adhesion. ICAM-3 is a ligand for DC-SIGN which is present on dendritic cells. Their exact function in exosomes is not very clear, although ICAM-1 has shown to be important in the adhesion of exosomes to immune cells expressing LFA-1 [66]. Even though several adhesion and targeting molecules, such as phosphatidylserine, thrombospondin-1 and tetraspanins, are known to be involved in receptor-mediated endocytosis, the precise mechanism of exosome interaction and internalization is still unknown [64]. However, data suggests that internalization is mainly achieved via endocytosis [78]. Exosomes derived from glioblastoma (GBM) cells show an uptake that is mainly regulated by non-clathrin dependent, lipid-raft mediated endocytosis. CAV-1, a protein involved in caveolae-dependent uptake, has been found to play a significant role in this type of lipid-raft mediated endocytosis. It negatively regulates the exosomal uptake by stabilizing lipid rafts and interfering with ERK1/2 signalling [78]. SKOV3 ovarian carcinoma cells are found to internalize exosomes via energy-dependent processes; clathrin-dependent endocytosis, macropinocytosis and a cholesterol associated pathway [79]. However, exosomes have also been shown to use fusion as an uptake route [80]. Parolini et al revealed that exosomes derived from melanoma cells are able to fuse directly with the plasma membrane of the recipient cell, and that the involvement of membrane proteins was only marginal. This implicates that the fusion process most likely depends on exosomal lipids and not its membrane proteins. Lipid rafts and externalized phosphatidylserine are implicated in facilitating fusion events, and have been found to be utilized by several viruses for fusion. Sphingolipids present in the lipid rafts, play a key role in conformational changes of fusion proteins, leading to fusion of two membranes [81]. The exosome bilayer contains both lipid rafts and externalized phosphatidylserine, indicating a possible fusion-function for these moieties in exosomes as well [75]. The tetraspanins (CD9 and -81) are also implicated in membrane fusion events [83]. CD9 is present in the surface of dendritic cell-derived exosomes and is involved in the direct fusion of the exosomal membrane with the target cell membrane [84]. Parolini et al also found a regulating role for the extracellular environment in the uptake and release of exosomes. An acidic environment increases both exosome uptake and release by melanoma cells, where the acidity is most likely the driving factor behind the traffic of exosomes within tissue and tumour mass [80]. As known, tumor cells have the ability to grow under acidic conditions. In order to see the effect of an acidic environment on exosome entry in tumors, inhibition of proton pumps was induced. This resulted in decreased exosome entry in tumor cells, indicating a role for the acidic environment in the internalization process. The internalization of exosomes by tumor cells mostly depends on lipid-mediated fusion, and increases when occurring in an acidic environment. The increased number of fusion events of exosomes is associated with an increase in exosomal membrane rigidity that is in turn related to the increase in sphingomyelin/ganglioside GM3 lipids. It was also found that acidic exosomes fuse better with acidic cells than buffered exosomes fuse with buffered cells. The explanation the authors offer is that the high numbers of GM3 lipids in the exosomal bilayer result in a highly negative particle, and when released in an acidic environment (high H⁺ content), the particle becomes positively charged and is able to fuse better to cells.

Lipids

Only a several number of the total variety of lipids in the EV bilayer has been investigated. These include the four prostaglandins (E₂, F₂, J₂, and D₂) and the conical lipid lysobisphosphatidic acid. And as lipids are important for the integrity of the EV's, these five investigated ones have shown to possess more than this 'singular' function [66, 85]. The four types of prostaglandins found in exosomes seem to be involved in intracellular signalling pathways that regulate cell physiology, and

are likely not involved in exosomal integrity. Lysobisphosphatidic acid is implicated in facilitating fusion of endocytosed EV's. Moreover, certain viruses interact with lysobisphosphatidic acid for endosomal escape. However, its function is likely limited to these processes combined with its involvement in EV budding.

The majority of the lipids in the bilayer are composed of membrane lipids also found in the plasma membrane, including sphingomyelin, phosphatidylcholine, phosphatidyl-ethanolamine, phosphatidylserine, ganglioside GM3, and phosphatidylinositol. The ratios in which these lipids are present in the bilayer depend on the parent cell, but several lipids (sphingomyelin, cholesterol, GM3, and phosphatidylserine) are enriched in all exosomes, independently of their origin [66, 86]. It also appears that the lipid composition is adapted to the microenvironment of the target cell. The pH of these environments might differ, and the rigidity of the EV must be maintained during these circumstances. Exosomes released in an acidic environment show higher concentrations of sphingomyelin and GM3 compared to exosomes released in a more buffered environment [80]. These lipids, upon interaction with cholesterol, are implicated in the increase of exosomal rigidity and stability. Furthermore, an increase in fusion was observed when these identical exosomes were released in an acidic environment compared to those released in a buffered microenvironment.

Even though lipids are very important for the biological function of EV's, it is not known whether they have any involvement in the process of target cell attachment.

Other factors

Recently, it has been described that heparan sulphate proteoglycans (HSPGs) play an important role in exosome internalization [88]. They are a family of proteins expressed on the surface of cells that are substituted with glycosaminoglycan (GAG) polysaccharides, which are sulphated. The sulphation largely determines their functionality. Internalized exosomes have been found to reside in vesicular structures that also contain syndecan and glypican, two classes of the cell surface HSPG's. Moreover, internalized exosomes down-regulate the surface expression of HSPG's. These findings suggest that HSPG's are used as internalization receptors, and not merely as attachment factors. Interestingly, viruses such as Herpes simplex virus-1 and lipoproteins also use HSPG's for cell entry and attachment [89]. Other factors such as lactadherin, acts as a scaffolding protein forming a bridge between phosphatidylserines present on target cells and integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ present on exosomes, thereby facilitating adhesion and uptake by cells expressing those specific integrins [66]. In some cases, exosomes do attach to the target cell, but are not internalized and yet they are able to induce changes in target cell homeostasis. Interaction of exosomes with surface receptors of macrophages was enough to induce IL-1 β mRNA expression. Full internalization of exosomes resulted in the induction of transcription of other mRNAs [90].

4 Virus- and EV-inspired nanotechnology

Over the years, the nanotechnology field has steadily gained more attention by various research fields, which has resulted in the development of new formulations and insights. Much has been learned through research with non-biological components that has led to a better understanding of particle behavior in the mammalian system. These lessons have been incorporated into the next generation of synthetic nanoparticles, but have also been of influence on the formation of biological particles. In this section, the major contributors that led to increased immune evasion, transport across barriers and specific target cell attachment and entry of VLP's and EV-inspired nanosystems are discussed. Moreover,

Lessons learned from non-biological systems

One of the major hurdles in the development of nanoparticle drug-delivery systems is the fast clearance rate induced by the innate immune system. This hampers the effective delivery of drugs to diseased tissue. New and improved drug-delivery systems with a prolonged circulation time and a short half-life to ensure drug release in a controlled manner without detrimental effects are needed [7]. The major contributors to the clearance reside in the reticuloendothelial system (RES) comprised of the liver, spleen and lungs. This system consists of phagocytic cells such as macrophages that engulf foreign or pathogenic particles, and subsequently clear them from the body [91, 92]. The fact that particles are cleared from the body via this system is not the actual problem. The real difficulty is that the majority of injected particles is cleared, leaving only a few to participate in the therapeutic effect [93]. Several attempts have been made to reduce the uptake by the RES. Polymer coatings seem to contribute positively to the circulation time of nanoparticles. Other characteristics such as size, shape and charge are also implicated in the process. However, only increasing the circulation time of nanoparticles is not enough. A balance between the accumulation and penetration of nanoparticles within target cells on one hand, and clearance by the RES on the other hand, must be found [2].

The morphology and surface chemistry of a particle greatly influence tissue distribution and clearance by the RES [7]. They can control the degree of particle self-interaction and particle opsonisation. Once a particle enters the systemic circulation, accumulation via different mechanisms occurs in various organs. For particles larger than 5-7 μm , entrapment in the smallest capillaries of the lung is especially relevant [91]. Particles that are smaller, but larger than 80 nm, accumulate in the organs of the RES [91, 92]. Still, if the clearance rate is low, particles can accumulate in other tissue that contains fenestrated capillaries through passive diffusion. Here, particles can escape through the fenestrated discontinuous endothelium and enter the extracellular space. Particles <80 nm can pass through the fenestrae in the liver and interact with the hepatocytes which can take up a large fraction of the injected dose. Very small, spherical particles (< 5.5nm) are mainly excreted via the kidney [92]. However, particles as large as 10-20 nm, such as dendrimers, quantum dots and gold nanoparticles, have shown to cross the endothelial layer and accumulate in the glomeruli of the kidney, where they are rapidly excreted into the urine as well [91]. Larger particles (>5.5nm) show variable behaviour in their pattern of distribution. This depends on their size, shape, rigidity, charge and flexibility [92].

Virus-like particles usually do not exceed the size of the native viral form. Modifications of the surface area, such as the addition of ligands or polymer coatings do increase the size of the particle,

however, this increase does not result in significantly larger particles. The size of particle does not only influence the distribution pattern, but also the rate of its clearance. As discussed previously, smaller particles evade internalization by macrophages more efficiently than larger particles. Research in pulmonary macrophages showed a 0.1% clearance of 20nm TiO₂ nanoparticles after 24 hours of exposure, whereas a 100% clearance of micrometre particles was achieved after the same period of time [18]. Mitragotri et al [19] showed that particles as large as 2-3 μm have the highest attachment to macrophages. The precise function of particle size during the internalization process is nicely described in [18, 15]. Here it is described how the dimensions of a particle are of importance for internalization. The size of a particle not only influences internalization, it actually determines the internalization rate and whether the internalization will be completed [18, 15]. Another factor involved in this is the particle shape. This is involved in the decision of a phagocytic cell to initiate phagocytosis; if a certain shape is not favoured, phagocytosis will not occur [18, 15]. A study performed in 1976 [95] was one of the first to show the effect of target size and shape on phagocytosis. Here, it is shown that phagocytosis of long fibers (asbestos and glass fibers) is delayed and/or remains incomplete. Since then it has been described that the reason for this lies in the local curvature of the particle, also known as the aspect ratio [15, 19]. In non-spherical particles, the aspect ratio determines the outcome of phagocytosis. Interaction of the phagocytic cell with the highest curvature of the target will result in the maximum number of phagocytic events. Also, studies with inorganic particles show that spheres were more phagocytised than rods or needles, and that spherical particles are internalized faster than ellipsoid particles [96]. Discoidal particles have also shown to have a higher circulation time than spherical particles, likely due to their drift towards the vessel wall [91]. Spherical, quasi-hemispherical and cylindrical particles, with a similar aspect ratio, are more easily internalized [91]. Filomicells, micelles consisting of degradable block copolymer amphiphiles that form filamentous particles, exhibit a longer circulation time and evade phagocytosis better than their spherical counterparts [20, 97]. Filomicelles have even been found to persist in circulation up to 1 week after intravenous injection, which is ten times longer than their spherical counterparts [98]. According to these and other results, small, non-spherical particles are more effective in the evasion of phagocytosis than larger and/or spherical particles. However, spherical particles are still used extensively, also within the virus-like particle field. This is mostly due to the fact that many viruses have a spherical native form.

The surface charge of a particle also influences the circulation time. Studies with liposomes show that small neutral vesicles have a higher circulation time than their anionic equivalents [7]. The neutral vesicles are less efficiently cleared by macrophages (Kupffer cells) due to less efficient opsonization compared to anionic particles, and are therefore poorly recognized by phagocytic cells. However, increasing the size of a neutral vesicles (> 100nm) also increases the clearance rate by Kupffer cells, indicating that aspect ratio still plays a significant role in complement opsonin adsorption [7]. For particles to escape splenic clearance, a particle must be small or flexible enough to evade splenic filtration [7]. The openings in the filtration meshwork present in splenic sinusoids rarely exceed 200-500nm in width which means that nanoparticles should not exceed the 200nm limit [7]. On the other hand, when targeting the spleen, particles should be more rigid and larger than 200nm.

4.1 Immune evasion

Virus-like particles: particle morphology

Animal virus-based VNPs such as adenoviruses particles, form icosahedral particles, have a relatively short circulation time (10-15 min.), and are rapidly cleared by phagocytic cells [4]. Polymer coating of the viral particles results in an increase in circulation time, however, adenoviral particles have shown to induce toxic effects including liver injury and acute induction of host immune response, raising concern for the use of these kinds of VNP platforms [4]. VNPs derived from bacteriophages and plant viruses are considered to be safer than their animal counterparts, as they are believed not to infect or replicate in mammalian cells and are therefore less immunogenic [2]. Animal studies with the Cowpea mosaic virus (CPMV) and Cowpea chlorotic mottle virus (CCMV) showed no toxicity towards the host, even though both viruses showed a wide distribution to a variety of tissue [2, 3]. Both viruses are spherical, a characteristic that could participate in the wide distribution effect observed. Research with rod-shaped TMV particles, CPMV particles and CCMV particles showed that indeed, the shape of a particle contributes to the distribution pattern [20]. TMV shows a preference for accumulation primarily in the spleen, while the spherical counterparts (CPMV and CCMV) showed a preference for the liver. However, the size, charge and ligands present on the outer surface of the particle also contribute to the tissue distribution pattern. Therefore one cannot state that this difference is due to the shape of the particle. Research performed with spherical TMV and rod-shaped TMV did not show large differences in tissue distribution between the two differently shaped TMV particles [22]. However, it was observed that the spherical TMV had a shorter circulation time, which was accounted to the fact that the spherical TMV only consisted of protein-protein interactions, which are somewhat weaker than protein-nucleic acid interactions, and therefore resulted in faster degradation of the particle. However, a fast clearance rate is not necessarily a negative characteristic. In fact, a somewhat fast clearance of a particle is necessary, as this would reduce detrimental side-effects. CCMV also has a fast clearance rate and has been widely studied as a drug-delivery platform [99]. However, CCMV has been found to evoke a strong immune response and after repeated administration some adverse effects were observed [100]. Polymer coating (PEG) of the particle is therefore a necessary step [99, 3]. CPMV particles have a high clearance rate and accumulate mainly in the liver, without any observed toxicity [4]. However, CPMV is known to be immunogenic. PEG coating of CPMV resulted in inhibited anti-CPMV response, increased circulation time and decreased liver and spleen uptake [100, 99, 4]. It also reduced epithelial cell interaction, by preventing interactions with surface vimentin proteins [101, 102]. The interaction between CPMV and vimentin might also explain the rapid clearance of the virus from circulation, where interaction might induce uptake into epithelial cells and thus reduce the amount in circulation [4].

Virus-like particles: particle surface chemistry

CCMV and CPMV particles have a negative surface charge, and are cleared faster (half-life is <15 min.) than phage Q β particles, which have a positively charged surface (half-life is > 3 hours) [2, 3, 4]. The surface charge properties of a VNP can be modified via chemical modifications and genetic engineering. Lysine side chains are often used for these modification techniques, and can be used to alter the surface charge of a VNP [2]. Coating particles with polymer chains has already been briefly mentioned. It is another method of altering a particles surface charge. Most coating polymers are composed of polyethylene-glycol (PEG) and its derivatives [7]. PEG is a neutrally charged, hydrophilic polymer that is non-toxic, and has a low degree of immunogenicity and antigenicity [2, 7]. It reduces biospecific interactions, resulting in a reduction of immunogenicity of the coated particle. Moreover,

PEGylated particles show increase in solubility and stability and thus also an increase in circulation time [22, 91, 2]. Once coated with PEG, particles show reduced opsonisation of serum opsonins, which are due to the provided neutrally charged hydrophilic layer [22, 103, 93]. However, PEGylation does not prevent the formation of a bio corona entirely, an acquired layer of plasma proteins and lipids, depended on the surface properties of the particle [91, 105, 93, 92]. The different surface characteristics, size and morphology of a particle attract different types of opsonins [7]. Subsequently, macrophages recognize the opsonized particle and sequester them in the liver, spleen and/or bone marrow [92, 7]. The specific distribution of particles and their clearance rate might be regulated by the different array of opsonins a particle collects [7]. Flow chamber experiments also revealed that opsonization is also influenced by the size of a particle [91, 3]. Smaller particles (< 100nm) accumulate less opsonins than larger particles, and the strength of the opsonization process increased once the diameter of the particles increased as well. PEGylation resulted in the decrease of this process and experiments performed with PEGylated VNPs such as CPMV, Potato virus X (PVX) and TMV are promising. PEGylated CPMV, with less than 1% coverage, reduced cell interactions in vivo and in vitro, and prevented initial immune response [2]. Similar results have been obtained for PEGylated TMV; increased circulation time for PEGylated TMV, compared to un-PEGylated TMV [22]. PEGylation not only improves circulation time, but it also leaves a large surface area and attachment sites available for additional modifications [2]. However, as beneficial as PEGylation seems to be to particle life-time, there have been reports that repeated administration of PEGylated particles induces an innate immune response, thereby undoing the initial PEG-effect [106]. Due to this, alternatives to PEG are being developed. Hu et al [107] created an innovative method where polymeric nanoparticles were coated with membrane lipids and proteins derived from erythrocytes, resulting in a higher circulation time compared to particles coated with PEG [107]. However, as promising as these results are, the versatility of the method and whether specific ligands could be introduced into the bilayer membrane remain to be elucidated.

Exosome-inspired nanoparticles

Exosomes are able to travel long distances when secreted into the bloodstream, and somehow are able to protect themselves from degradation [108]. Repeated intravenous administration of autologous exosomes into mice does not result in an immune response and administration of human derived exosomes into immune-competent mice is surprisingly well tolerated [109]. However, it is not known what effect repeated administration has in that setting and whether non-self exosomes are tolerated in humans as well. Clinical trials using autologous exosomes loaded with cancer antigens have shown that repeated administration of self-exosomes is well tolerated by patients, again underlining the lack of immune response by exosome administration.

Exosomes have also been shown to actively suppress immune activation. Exosomes derived from the placenta express Fas ligand (FasL), a transmembrane protein involved in the regulation of the immune system, on their surfaces that T-cell activation [110]. The same ligand, when present on exosomes derived from activated T-cells, is also involved in the induction of T-cell apoptosis.

4.2 Exploiting natural pathways for transport across barriers

Virus-like particles: particle morphology

Like viruses, intravenously injected nanoparticles need to cross the endothelial barrier to deliver their contents to the target cell. And as the endothelial barrier is a tightly regulated border, particles must be either small enough to be transported paracellularly, or use existing transport mechanisms to get across. These can include a passive or an active process. Passive extravasation of larger particles can only occur through vessels containing fenestrae. These are mainly found in the sinuses of the liver or at sites where the endothelial integrity is compromised as often seen in tumor vasculature or inflamed tissue [7]. The fenestrae of liver sinusoids can be as large as 150nm, however, the majority has a size of <80nm. This enables the majority of nanoparticles to cross the endothelial barrier in a passive manner in these types of tissues. Even highly flexible particles of approx. 400nm can pass through these pores. Openings of the discontinuous endothelium of tumor vasculature or inflamed tissue can even reach a diameter of 700nm [7]. The tumor vasculature of many solid tumors is a highly permeable system and enables the enhanced permeation and retention (EPR) effect, where particles (< 500nm) can passively extravasate of the tumor vasculature and accumulate in the tumor milieu [91]. The fact that the majority of solid tumors portray this effect makes them an attractive target for drug-delivery systems. Drugs that are highly potent and have a small therapeutic window could benefit from drug-delivery systems that utilize this effect. However, once in the interstitium, extravasated particles do not travel far into the tissue, due to high interstitial pressure. Moreover, a tumor often consists of a highly perfused, fast growing, outer layer combined with a low perfused, necrotic core. Low perfused areas do not show the EPR effect and are therefore less exposed to the drugs than highly perfused areas, thereby limiting the efficacy of the drug-delivery system [7].

Virus-like particles: particle targeting moieties

Targeted drug delivery requires targeting moieties that should increase the cell-specific targeting of a particle. However, the presence of these surface decorations might hamper the transport across barriers. Moreover, the transport across these barriers is considered to be the rate-limiting step of the delivery systems, and is thus an important hurdle [91]. Usually, particle extravasation is restricted to sites with fenestrations, compromised endothelial integrity due to inflammation or via the EPR effect found in some tumors. Unfortunately, not much is known about extravasation of viral-nanoparticles. CPMV is known to be internalized by endothelial cells, however, this does not result in subsequent release on the other side. Also, the majority of viral nanoparticles that are used as drug carriers are comprised of plant viruses, of which the majority does not interact with mammalian cells. And as the transport across endothelial barrier requires receptor-mediated interaction, targeting moieties should be incorporated within the VLP in order to get the wanted effect.

Exosome-inspired nanoparticles

Specific crossing of the endothelial barrier is a problem for all delivery systems, even the naturally occurring ones. However, recent experiments have shown that with the use of fusion proteins, it is able for exosomes to cross the blood-brain barrier and release their cargo into brain cells [111]. RVG is a ligand for the alpha-7-subunit of the nicotinic acetylcholine receptor that is present on the endothelial lining of brain vessels, neurons and neuro-muscular junctions, and is likely involved in transcytosis of exosomes. Incorporated fusion proteins displaying the RVG ligand have shown to possess an ability to cross the barrier and deposit their cargo in neuronal cells. The small size of an exosome could also be beneficial for the transport across the endothelial barrier, as the small size of

a particle facilitates the extravasation through fenestrations present in certain vessels, a process that is often seen in liver and some tumors [84]. However, this type of extravasation is not controlled and in some cases not wanted as the exosomes, like nanoparticles, can end up in a variety of tissue.

4.3 Addition of ligands and other moieties improve targeting of biological nano-particles

Nanoparticles can be created in such a way that they display a certain synthetic tropism, where ideally, intravenously injected particles display a preference for diseased tissue that exceeds the preference for healthy tissue. Targeted drug-delivery could increase the specificity of the drug-delivery system, and thereby reduce toxicity to non-targeted tissue. It could also increase the amount of drug delivered to the target tissue, thereby reducing the initial amount given to the patient. The use of nanoparticles for therapeutic or diagnostic purposes requires functionalization of the particles with specific molecules (e.g. peptides, ligands, chemical groups, receptor-binding domains or sequences and bifunctional crosslinkers) to increase specificity [93, 112]. Although the specificity of these particles is not at the level of those of viruses, current technology has developed new methods and techniques that highly improve the functionality of current drug-delivery systems. Covalent attachment of ligands to viral capsids is a sensitive process. The self-assembly of altered capsid proteins is prone to deleterious effects after modifications. In spite of this, the covalent attachment of ligands to viral capsid proteins is promising, and the variety of target proteins and peptides that can be used for this application has expanded [5]. These include proteins involved in cell-cell or cell-matrix interactions (such as E-selectin, vascular cell adhesion molecule-1, and the $\alpha\beta3$ integrin complex) and growth factors receptors [7].

Virus-like particles: particle morphology (target cell attachment)

The benefit of VLPs is that they can be easily modified. Lactobionic acid, glycopolyomes, human holo-transferrin, folic and folate acid, and antibodies are a number of biomolecules that have been used in research towards targeted drug delivery [99]. Many VLPs contain reactive groups that make them susceptible to modification. And currently, the research focus lies mostly on plant viruses, which is mainly attributed to the fact that they are considered to be safe. CPMV for example, can be readily modified due to its surface lysines [101, 113]. It is also highly stable, even under harsh conditions, making it an attractive candidate as a drug-delivery vector [4]. CPMV has been extensively researched in its potential for targeted drug delivery to solid tumors. Coating the particle with a conjugation of short PEG chains and folic acid (FA; ligand for folate receptor and overexpressed in many types of tumors), resulted in specific targeting to cancer cells [101, 99]. Interestingly, FA directly conjugated to CPMV coat protein did not achieve this effect, but FA directly conjugated to HCRSV and *Red clover necrotic mottle virus* (RCNMV), did result in positive tumor targeting results [114, 2]. Also, direct conjugation of folate acid, a moiety similar to FA, to an adenoviral particle also showed positive effects [115]. These results indicate that folic and folate acids increase tumor specificity of viral nanoparticles, however this effect is not uniform. In humans, CPMV targets surface vimentin, a protein found on a variety of cells. Interestingly, vimentin is especially found on endothelial cells lining the tumor vasculature, which makes CPMV naturally attracted to these cells [63]. CPMV usually does not extravasate from the vasculature, however, due to the highly permeable structure and the overexpression of vimentin, CPMV does extravasate from tumor vasculature.

However, whether a plant or animal virus is more suited as a drug delivery system depends on the cargo, target and application. Gene therapy for example, is best achieved by using animal viruses as these viruses are able to infect mammalian cells and incorporate their own genome into that of the host. And animal viruses such as adenovirus, retrovirus, adeno-associated virus (AAV) with modified genomes, have been widely used as targeted delivery system for gene therapy [5]. Adenoviruses in particular, have been studied very extensively and over the years. Genetically modified strains have been developed that possess low immunogenicity and toxicity and simultaneously exhibit high infectivity [116]. Also, due to their natural tropism for specific cells, targeting can be more readily achieved. The specific tropism can also be altered by introducing peptides targeting to other cells or by coating the particle and preventing the original interaction [1]. Adenoviral VLPs consisting of only the capsid protein are still able to bind to the adenoviral associated receptor CAR. However, as this receptor is not upregulated in cancer cells, the interaction of AdV VLPs with cancer cells is low. Coating these particles with PAMAM dendrimers conjugated with GE11, a targeting peptide for EGFR, increases the binding avidity of the particle for cancer cells and increases internalization by cells expressing low or medium levels of CAR, compared to uncoated particles. Also, the coating results in a charge switch of the particle, making it a positive particle instead of a negative one, which could be beneficial as the surface charge of mammalian cells is negative [118]. However, these formulations are impaired as there is a lack of knowledge on the structure-function relationships of viral peptides and target cell receptors. Another way of altering the original tropism of a virus-like particle (AdV) is to alter the self-assembly process. Adenoviral particles usually consist of 12 pentons (formed by 12 penton bases and 12 penton fibers), however, research has shown that the fiber protein is not necessary for self-assembly, but is needed for interactions with CAR. The penton base is capable of forming a particle on its own [119]. The penton base particle (DB) is somewhat smaller and enters cells via a heparin sulfate specific manner, unlike the original particle. It is successful in cancer drug delivery to diseased cells and has a 100 times higher bioavailability compared to microspheres loaded with the same drug.

Another extensively studied animal virus is HCV. A study performed on fused HCV core proteins with RGD (arginine-glycine-aspartic acid) peptide (a ligand for the $\alpha_v\beta_3$ integrin receptor which is an overexpressed surface receptor on many types of cancer and tumor neovasculature) combined with IFN- α 2a resulted in inhibition of breast cancer cell migration and invasion [120]. The self-assembled HCV particle consisting of the HCV core protein, can bind non-specifically to a large variety of cells. Genetically deleting these areas disrupts the nonspecific binding, without disturbing the self-assembly process [121]. Moreover, insertion of several ligands into a specific area of the protein (78-81 AA) has shown not to alter the proteins structure [121]. This enables the insertion of for example a Z_{HER2} affibody (recognizes and binds to human epidermal growth factor receptor-related 2 (HER2)) into this region of the core protein, and result in specific targeting of HER2-expressing breast cancer cells. Studies with the human JC polyomavirus (JCV) have shown that JCV is an effective carrier of anticancer drugs. Niikura et al [122] created a glutathione (GSH)-triggered release system incorporated in JCV particles, where cytoplasmic GSH induces a redox-responsive drug delivery into the cytoplasm. This system showed to be more effective as encapsulated PTX showed a higher toxic activity in lower concentrations than free PTX did. Although JCV is an animal virus, the particles formed out of the VP1 coat protein do not exhibit any cell specificity, and thereby could be very toxic to healthy tissue. However, the trigger-release system is very promising. Another polyomavirus, simian virus 40 (SV40), has also been extensively investigated. It has high blood compatibility and exerts a low toxicity in cells [123]. Conjugation of hEGF to the VP1 coat protein of SV40 resulted in

increased targeting towards EGFR overexpressing cancer cells and subsequent internalization via EGFR-mediated endocytosis.

Genetic engineering has resulted in the development of new types of surface ligands; bifunctional crosslinkers, which bind both to the vector and to a cell-surface receptor, combined with receptor-binding domains results. [112] investigated the functionality of genetically modified polyomavirus capsids with an inserted WW domain (that binds proline-rich sequences; PLPP consensus motifs) that can bind a PLPP tagged protein, such as an antibody or receptor-binding domain. Even though the insertion of the WW domain did not disrupt the integrity of the viral capsid structure as it was inserted in a highly variable region of the VP1 capsid protein, the fast dissociation reaction of the PLPP containing protein would result in reduced effectiveness of the bound ligand in vivo. Even though this method is not optimal yet, this system has the ability to expand the usefulness of a particle as the targeting moieties could be interchangeable.

Exosome-inspired nanoparticles: particle targeting moieties

Extracellular vesicles can be manipulated in such a way that they can express specific proteins on their surface. Alvarez-Erviti et al were the first to incorporate a RVG moiety into the exosomal bilayer and deliver macromolecular drugs into the brain [111]. They engineered murine exosomes to express an exosomal membrane protein Lamp2b fused to the RVG targeting peptide that were shown to specifically deliver siRNA into neuronal target cells after intravenous injection. The authors also tried to deliver drugs to muscle cells using a muscle specific peptide of which the efficacy was already determined. However, unlike the neuronal targeted exosomes, the muscle targeted exosomes were not able to deliver their cargo to muscle cells. And even though the muscle specific peptide was effective in combination with cell penetrating peptides and antisense oligo's, it did not result in similar effects when combined with exosomes. This shows that ligands are not interchangeable between systems and that one cannot assume that the effectiveness of a ligand in a specific system will resemble the effectiveness of the same ligand in another system [84]. These modified exosomes do not elicit any short term innate immune response [111]. However, whether repeated administration induces and adaptive immune response is not known [84]. Another study incorporated a fusion protein consisting of platelet-derived growth factor with hemagglutinin, myc-tag and a targeting peptide (EGF or GE11) that was successfully expressed on the surface of exosomes. The resulting vesicle was able to target tumor cells overexpressing EGFR without eliciting a response [124].

Targeting of exosomes to specific cells can also be achieved via a ligand-independent mechanism as demonstrated by [65]. Here, targeting of curcumin-complexed exosomes to activated myeloid target cells was achieved by exploiting the behaviour of activated myeloid cells. Monocyte-derived myeloid cells are involved in inflammation-related immune diseases and cancer. They act as scavengers and potential effector cells during inflammation. However, uncontrolled activation of myeloid cells results in chronic inflammation. But activated myeloid cells also display increased scavenger activity, and should therefore also have an increased uptake of exosomes than inactivated cells. Indeed, activated myeloid cells show more curcumin-complexed exosome uptake than inactivated cells and therefore also an increase of induced apoptosis due to the higher effectiveness of curcumin. Here, the exosomes were not genetically altered, nor were the recipient cells, but the diseased state of the recipient cells was utilized in such a way that therapeutic effects were achieved.

5 Additional lessons to be learned?

Virus-inspired and EV-inspired nanotechnology have shown to be promising tools for the treatment of various diseases and in particular cancer. However, more research in both fields is needed in order to develop a particle that is both effective and safe in its use as a targeted drug-delivery system. And this research does not limit itself to the discussed topics. Unfortunately, we cannot discuss all challenges that these systems still face, as this would be a thesis on its own, however, we can discuss how some of the characteristics used by natural nanoparticles that could be incorporated into the 'man-made' systems.

Characteristics that could aid in enhanced immune evasion

One of the difficulties biological nanoparticles still encounter is the innate immune system. Especially repeated administration seems to be a hurdle. Enveloped viruses such as HIV-1 contain high number of glycosylated glycoproteins that function as a shield and protect them from detection by immune cells [39]. One might consider incorporating these types of proteins into VLP's. There has been some research on this topic where synthetic nanoparticles were coated with heparin, a ligand for factor H that is a negative regulator of the complement system, and prolonged circulation was achieved upon intravenous injected mice. However, this effect was unlikely due to effective inhibition of the complement as murine Kupffer cells lack complement receptors with scavenging ability and complement inhibition was only demonstrated in human serum. It is more likely that the prolonged circulation time was due to the high hydrophilicity of the particles. Nonetheless, incorporating ligands expressed on the surface of either biological or synthetic nanoparticles could be beneficial for immune evasion. Incorporation of sialic acid into the outer layer could also provide some benefit. Liposomes expressing sialic acid containing gangliosides were able to effectively inhibit complement activation, however, incorporation of the same ligand into synthetic nanoparticles resulted in less promising results [7].

A different approach is to disguise nanoparticles with 'self-peptides'. CD47 is present on the surface of erythrocytes and lymphocytes and protects them from degradation by interacting with SIRP α that result in inhibition of phagocytosis. Several viruses including smallpox and vaccinia display analogues of this protein and thereby exploit the intrinsic mechanism [125]. Incorporation of CD47 into VLP's might enhance their circulation time.

There are numerous of different ligands that could benefit VLP circulation, however, investigating their behaviour and efficacy is a time-consuming process.

Characteristics that could aid in enhanced transport across barriers

Specific transport across the endothelial barrier is the rate-limiting step of intravenously administered nanoparticles. This is mainly attributed to the fact that it is quite difficult to control this process. There are two ways in which a particle can cross the barrier without infecting cells; via receptor-mediated transcytosis and paracellularly. However, the paracellular route is only reserved for particles smaller than 3nm or during inflammation where the tight junctions are disrupted. Some viruses (HIV-1) have developed ways of exploiting the mechanism of transcytosis by the binding of gp120 to the galactosyl ceramide that induces the formation of caveolar vesicles. Whether incorporation of gp120 into either viral- or EV-inspired particles would be helpful is questionable. The gp120 protein is a very active one; exposure of the protein to the epithelium disrupts epithelial tight

junctions and is able to induce transcytosis in the blood-brain barrier. Incorporation of this protein into nanoparticles will probably result in adverse effects. However, the idea of incorporating a moiety that facilitates transport is one to be explored. Albumin is also transported via transcytosis, and it uses the gp60 receptor as an internalization receptor. Perhaps it is possible to create a fusion protein containing the interacting domain of albumin that is able to induce transcytosis as well. This could be applied to both systems (EV-inspired and virus-like particles) as both can be modified and manipulated to expose preferred ligands.

Characteristics that could aid in enhanced targeting to specific cells

A lot of research is being done concerning the incorporation of targeting moieties that enhance nanosystem specificity. This is mostly focused on the incorporation of targeting ligands of different origins. One could consider the combination of viral characteristics with exosomes to enhance targeting [57]. Certain viruses hijack the exosome biogenesis route and modify the exosomes in such a way that they benefit the viral infectivity. By manipulating the virus that hijacks the route, exosomes with specific viral characteristics can be formed. Which would contain the benefits of exosomes combined with the selected benefits of viruses?

The specific targeting of exosomes is mainly regulated by the combination of specific tetraspanins. Once the specific combinations of tetraspanins are known, these exosomes could be exploited to enable targeting to specific cells. It might also be possible to incorporate these combinations of tetraspanins onto synthetic nanoparticles, which could also lead to enhanced targeting. Tetraspanins are also involved in exosomal entry into target cells, therefore it might be that the presence of tetraspanins on nanoparticles could also aid in cell entry.

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