INNOVATION

Artificial viruses: a nanotechnological approach to gene delivery

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Abstract | Nanotechnology is a rapidly expanding multidisciplinary field in which highly sophisticated nanoscale devices are constructed from atoms, molecules or (macro)molecular assemblies. In the field of gene medicine, systems for delivering nucleic acids are being developed that incorporate virus-like functions in a single nanoparticle. Although their development is still in its infancy, it is expected that such artificial viruses will have a great impact on the advancements of gene therapeutics.

With the completion of the Human Genome Project[1] we now have access to a wealth of information that has accelerated the identification of potential new targets for therapeutic intervention. As many diseases have a genetic basis, the use of nucleic acids (that is, DNA, antisense oligonucleotides and small interfering RNAs) to correct missing genes, replace defective genes or downregulate aberrant gene expression is an attractive strategy for treatment. However, despite the enticing promise of nucleic-acid-based therapies, their practical application has been disappointing. To date, more than 1,000 different gene-therapy clinical trials for the treatment of many different diseases are in progress worldwide (see Further information, Gene Therapy Clinical Trials Worldwide). Notwithstanding this large number of ongoing trials, success with gene therapy has been limited (TABLE 1) and has not yet resulted in approval of a gene therapy product by the US FDA. This lack of success can be ascribed to difficulties related to the effective introduction of nucleic acids into target cells, which requires the use of sophisticated delivery systems. For the introduction of foreign DNA, two different types of delivery systems can be distinguished: those that are manmade (with the exception of the plasmid DNA component, which is typically produced in bacteria) and those that are derived from viruses.

Synthetic gene-delivery systems consist of a self-assembling complex of DNA with positively charged molecules (for example, polymers, peptides, lipids or combinations thereof) (FIG. 1). These complexes are small in size (40–150 nm) and usually have a net positive surface charge, which enables adsorption-mediated cell binding and internalization. The popularity of synthetic vectors is mainly due to ease of preparation and use rather than their transfection efficiencies. In general, the transfection efficiency of the present generation of synthetic vectors is poor.

Conversely, viruses are adept at entering host cells. They have evolved cunning mechanisms to survive in the extracellular environment, attach to cells, cross cellular membranes, hijack intracellular transport systems and subsequently deliver their genomes into the appropriate subcellular compartment (for example, cytosol or nucleus). Due to this very efficient cell-entry mechanism, the transfection efficiencies of viral vectors remain unprecedented. However, these viral vectors have several major restrictions, such as limited DNA-carrying capacity, lack of target-cell specificity, immunogenicity and, for some viral vectors, insertional mutagenesis, which limit their use in clinical settings. The recent reports on the occurrence of leukaemia in three X-linked severe combined immunodeficiency (X-SCID) patients treated with a retroviral vector, in addition to a reported death caused by the injection of adenovirus particles into the hepatic duct of a patient with ornithine transcarbamylase (OTC) deficiency, during gene therapy clinical trials have reignited discussions about the safety of some viral vectors[2,3].

The ultimate challenge is to design an artificial carrier that equals (or even exceeds) its viral counterparts in terms of transfection efficiency, but which is safe to use, target-cell specific, non-immunogenic and relatively inexpensive to prepare at scales that permit clinical use. Designing and preparing such an artificial virus is difficult to achieve as it

Table 1 | **Some successful applications of gene-delivery systems in gene-therapy clinical trials**

Indication	Vector	Gene	Clinical Phase	Treatment	Results	References
Severe combined immunodeficiency (ADA-SCID)	Retrovirus	Adenosine deaminase (ADA)	I/II	*Ex vivo* gene transfer into autologous CD34+ cells	2/2 patients were cured with no significant adverse events	44
Melanoma	Lipofection	HLA-B7/ β_2-microgobulin	II	Intratumoral gene transfer	Regression of treated melanoma lesions was observed in 18% of the patients	45, 46
Malignant glioma, sarcomas, pancreatic carcinomas	Oncolytic adenovirus	–	I/II	Intratumoral gene transfer	No adverse events up to 10^{10} PFU, partial responses.	47, 48
Severe combined immunodeficiency (SCID)-X1	Retrovirus	γc cytokine receptor subunit	I/II	*Ex vivo* gene transfer into autologous blood stem cells	Restoration of immune function in 4/4 patients. No significant adverse events	49
Cystic fibrosis	Adeno-associated virus	Cystic fibrosis transmembrane conductance regulator gene [tgAAVCF]	II	Aerosol-mediated pulmonary gene transfer	No adverse events. Improved lung function in treated group. No detectable transgene expression	50

ADA, adenosine deaminase; SCID, severe combined immunodeficiency; tgAAVCF, transgenic adeno-associated virus containing *cystic fibrosis* gene.

requires the integration of many different and often counteracting features in a single particle. Artificial viruses should be stable and inert during transport through the body but, on encountering target cells, a cascade of events should be triggered that eventually leads to delivery of the associated DNA into the nucleus of these cells. The design of artificial viruses is a good example of nanotechnology, which involves the creation of a fully functional gene-delivery system through the manipulation and assembly of (macro)molecular structures. Here, we give our perspective of how artificial viruses can be designed *de novo* by assembling different functional components required for effective gene delivery into a single gene-delivery vector.

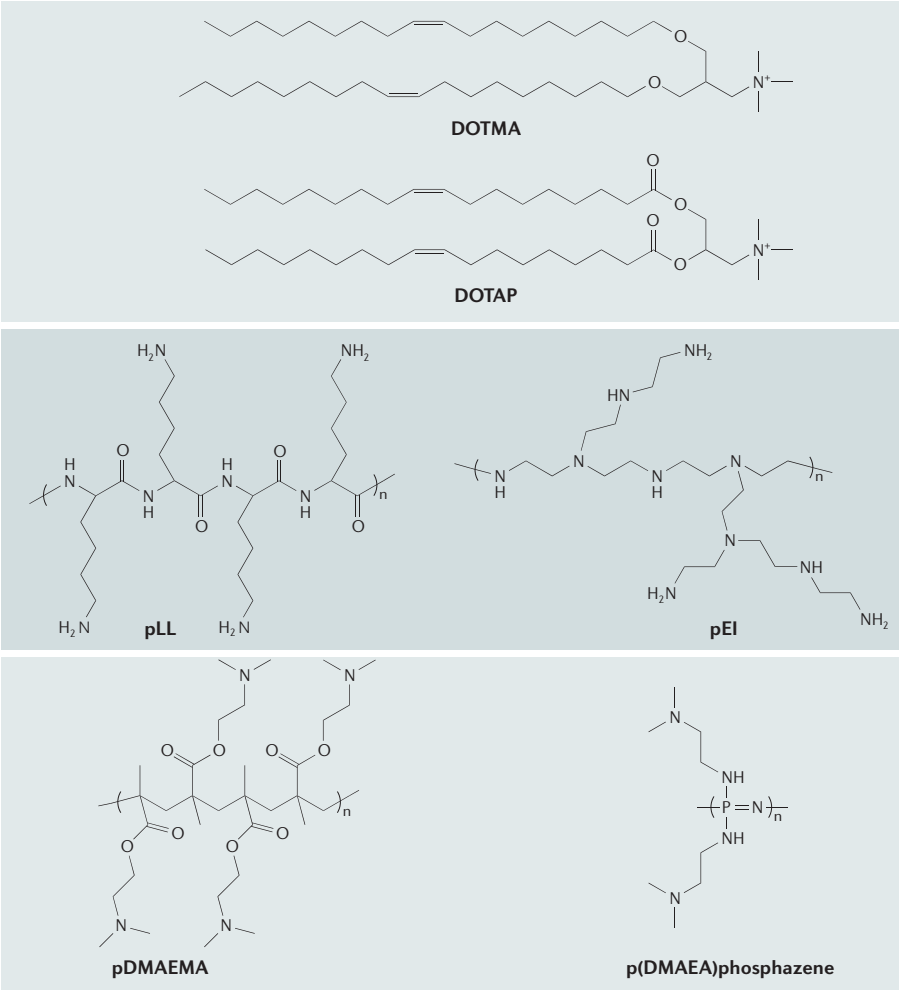

Figure 1 | **Commonly used cationic molecules for the condensation of plasmid DNA.** DOTMA: N-[1-(2,3,-dioleyloxy)propyl]-N,N,N-trimethylammonium choloride; DOTAP: 1,2-dioleoyl-3-trimethylammonium propane; pLL: poly (L-lysine); pEI: poly(ethylenimine); pDMAEMA: poly(2-(dimethylamino)ethyl methacrylate; p(DMAEA)phosphazene: poly(2-dimethylaminoethylamino) phosphazene.

Nucleic-acid delivery: the challenges

Efficient gene delivery is not an easy task as there are many biological barriers that need to be overcome (FIG. 2). In case of intravenous administration, the carrier is exposed to blood components. This can result in premature destabilization with concomitant release and degradation of the plasmid DNA. The half-life of naked plasmid DNA in blood is on the order of minutes[4]. Carrier-mediated protection during transport through the blood circulation is therefore a prerequisite to make the DNA inaccessible to degradative enzymes. Furthermore, carriers can be recognized as foreign and cleared from the blood circulation by cells of the mononuclear phagocytic system (MPS) before having the chance to encounter target cells within or outside the vascular system. MPS uptake can be enhanced by opsonization of the carrier with blood components such as complement factors or antibodies.

Local administration requires efficient distribution and penetration of the gene carriers into the tissue. Transport of gene carriers through the interstitial space of tissue is mediated by both diffusion and convection, and can be slowed down by nonspecific interactions with extracellular matrix components or high interstitial fluid pressures[5].

Irrespective of the route of administration, the gene carriers should be able to bind to cells to allow cellular uptake. This is not, in general, a problem if the gene carriers have a net positive surface charge, which readily induces adsorption onto negatively charged cell membranes. However, this form of binding is indiscriminate and does not allow restricted delivery to target cells (for example, tumour cells). Furthermore, binding should lead to internalization as a strategy to bring as much DNA into the cell as possible. This puts a limit to the maximum size of the DNA carriers as most cells, with the exception of specialized phagocytes, cannot efficiently internalize large particles (those >500 nm in diameter)[6].

Once inside the cell several intracellular barriers need to be crossed before the foreign DNA can be transcribed and translated (FIG. 2). The first intracellular barrier encountered is the endosomal compartment. The entrapment of internalized DNA carriers in endocytic compartments prevents further intracellular transport towards the nucleus, and will often result in degradation of the carrier and its associated DNA in the endosomal/lysosomal compartments. Those DNA carriers that manage to escape the endosomal compartments are then challenged by the complex environment of the cytosol, which contains many filamentous structures that impede the free diffusion of large particles such as DNA carriers. Dissociation of the carrier at this stage might be required to allow further transport of the freed plasmid DNA molecules. However, several studies have found evidence that plasmid DNA is largely immobile in the cytosol and, in addition, is rapidly degraded by cytosolic nucleases[7–9]. Free or carrier-associated DNA that reaches the nucleus intact is put to a hold at the cytosolic side of the nuclear envelope. The nuclear envelope is a double-membrane enclosure that prevents the free passage of large molecules from and into the nucleus[10]. Small molecules (<40 kDa) can diffuse freely through the pores of the nuclear pore complexes (NPC), whereas larger molecules and particles (up to 40 nm in size) can only be imported through the NPC by an active transport mechanism[11]. During mitosis, the nuclear barrier breaks

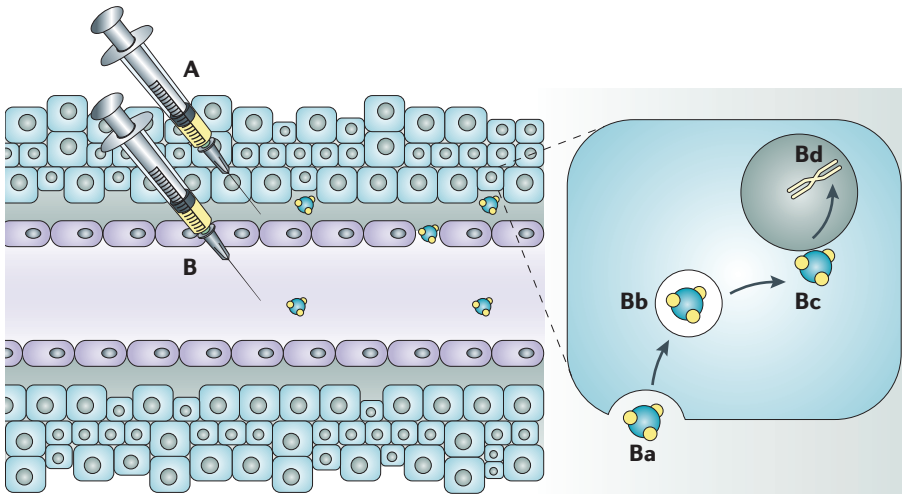

Figure 2 | **Extracellular and intracellular barriers to local and systemic gene delivery.** After local (**A**) or intravenous (**B**) injection, gene-delivery carriers are exposed to components and cells that can cause destabilization and removal of the gene carriers, respectively. To reach their target cells, locally injected gene carriers have to penetrate the interstitium either by convection or passive diffusion. For intravenously injected gene carriers, the carriers first have to extravasate from the circulation into the interstitium. Gene carriers can be cleared from the interstitium by lymph drainage, but those carriers that survive the harsh conditions should be able to recognize and bind target cells (**Ba**). After binding, gene carriers have to pass across the cell membrane or, after endocytosis of the carriers, the endosomal membrane (**Bb**). Inside the cytosol gene carriers have to be transported towards the nuclear envelope (**Bc**). Either the entire gene carrier or its associated DNA needs to pass the nuclear envelope to reach the nucleus, where the transgenes can be transcribed (**Bd**).

down, which explains the increased levels of transfection seen in dividing cells compared with their growth-arrested counterparts[12]. However, as most cells do not divide or divide only slowly, an active transport mechanism is needed to carry the DNA from the cytosol into the nucleus.

Inside the nucleus, the transgene encoded on the plasmid vector should be expressed to establish therapeutic levels of recombinant proteins within the affected cell. This requires gene transcription regulatory elements, such as promoters and enhancers, to drive the expression of the transgene in mammalian cells. Viral promoters are often used because of their strong transcriptional activation. However, their constitutive nature does not allow control over the level of transgene expression. For the expression of proteins with a narrow therapeutic window, tight control over the level of transgene expression is essential. In addition, the introduction of foreign DNA into mammalian cells can induce a profound immune response, presumably triggered by differences in the degree of methylation of the foreign DNA compared with the mammalian genome[13].

Artificial viruses: requirements

In light of the impediments to gene delivery it is evident that an artificial gene-delivery vector capable of performing all these tasks should be intelligently designed and will have a multi-component architecture, in which each component fulfills a different task in a timely and concerted fashion. The functional components that we think are needed for effective gene delivery and the requirements these components should meet are elaborated below.

Bio-compatible, bio-degradable and bio-invisible. The artificial virus should preferably be constructed from materials that are biocompatible and biodegradable to prevent carrier-induced toxicities and the accumulation of carrier components in the body. A selection of commonly used cationic molecules to condense DNA is given in FIG. 1. In general, lipids are well tolerated. Synthetic polymers, on the other hand, have shown to induce some cytotoxicity *in vitro*[14] and *in vivo*[15]. It is difficult to predict, however, which polymer will be cytotoxic and which not on the basis of the structure of the cationic polymer. In general, low-molecular-mass cationic polymers are less toxic than high-molecular-mass polymers.

Peptides derived from L-amino acids are inherently biodegradable. Nonetheless, when proteins or peptides contain large numbers of positively charged or exposed hydrophobic amino acids, they can destabilize biological membranes and thereby cause cytotoxicity[15].

In addition to biodegradability and biocompatibility, the artificial virus should be 'invisible' to the innate and acquired immune system of the patient in order to prevent unwanted immune reactions against the carrier and, consequently, rapid clearance of carriers from the blood circulation after intravenous administration. This can be achieved by adding a hydrophilic coat around the carrier. The coat can consist of a lipid bilayer[16] or hydrophilic polymers grafted onto the surface of the carriers. For instance, surface modification of the carrier with the hydrophilic flexible polymer polyethylene glycol (PEG) results in drastically prolonged circulation times of polyplexes after intravenous administration[17]. Biodegradable alternatives such as polyhydroxyalkyl L-asparagine/L-glutamine (PHEA/PHEG)[18] might also be considered.

Cell binding and internalization. A concomitant effect of the surface modification of gene carriers is that the positive surface charges are shielded, which drastically reduces the nonspecific adsorption onto cell membranes. This enables targeting of the gene carriers towards specific cell types by conjugating ligands to the hydrophilic coat around the gene carrier that specifically bind internalizing cell-surface receptors. In this way, delivery of the transgene and subsequent expression can be restricted to target cells. Several different types of targeting ligands have been used for this purpose, including peptides[19], antibodies[20] and vitamins[21]. If targeting ligands are directed towards internalizing receptors, receptor binding will lead to receptor-mediated endocytosis of the targeted gene carriers, provided they are small enough (<200–300 nm). This route of uptake is to be preferred as it guarantees intracellular accumulation of gene carriers in a receptor-specific way.

Endosomal escape. After cellular uptake of the gene carriers, the carrier should somehow escape the confines of the endosomal compartment in order to reach the cytosol and, from there, the nucleus. This requires dissociation of the internalized carrier from the receptors that triggered the internalization process. In addition, the membranes of the endosomes should be destabilized to allow translocation of the carriers into the cytosol. Synthetic peptides that mimick the amino-terminal fusion portion of the influenza virus hemagglutinin have been used to facilitate endosomal escape[22,23], but also entire proteins, such as listeriolysin O from *Listeria monocytogenes*, can be used to mediate endosomal escape[24]. However, the use of

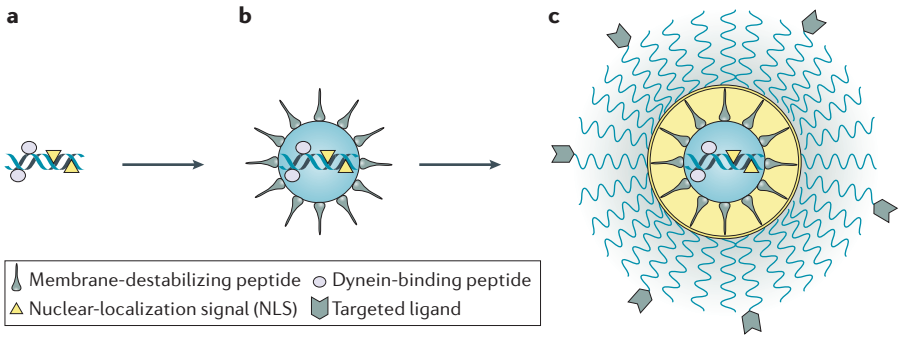

Figure 3 | **A conceptual model of the assembly of a multi-layered artificial virus.** Artificial viruses consist of a cationic core and an anionic shell. The cationic core is composed of pDNA to which functional peptides are bound with high affinity (**a**). These functional peptides (for example, dynein-binding peptides and peptides with nuclear-localization signal sequences) should facilitate the intracellular transport of DNA towards and into the nucleus once the DNA has been released from the core into the cytoplasm. The DNA is condensed into the core complex by low-molecular-mass cationic structures (for example, peptides or biodegradable polymers), which are cross-linked with reducible bonds for stability (**b**). Included in the core are membrane-active peptides that can destabilize endosomal membranes at low pH. The shell is attached or adsorbed onto the cationic surface of the core and consists of polar or negatively charged flexible polymers or peptides. This shell serves as a scaffold to which targeting ligands can be attached (**c**). Surface-exposed ligands should mediate cell-specific attachment that trigger internalization of the artificial virus by receptor-mediated endocytosis.

bacterial proteins will probably induce an immune reaction when injected in humans, and so small peptides are therefore preferred.

Cytosolic trafficking. The cytoplasm is composed of a fluid portion (the cytosol) in which a mesh-like network of microfilaments and microtubules (the cytoskeleton) and several different subcellular organelles are embedded. Both the cytoskeleton and the high protein content of the cytosol limit the diffusion of large structures such as macromolecules and intracellular vesicles. The diffusion of such structures is 500–1,000 times slower in the cytosol compared with diffusion in water[25]. Therefore, the cell uses different kinds of molecular motors to transport intracellular vesicles and large macromolecules, including mRNA through the cytoplasm. Dyneins, for instance, are molecular motors that carry cargo along microtubules in a retrograde fashion (that is, from the periphery towards the cell nucleus) and is used by adenoviruses, among others, to facilitate transport towards the nuclear envelope[26].

Similarly, artificial gene carriers or pDNA molecules can be actively transported towards the nucleus if they expose peptides or proteins that can specifically interact with the dynein molecular motor complex[27,28]. Such active transport will greatly reduce the cytosolic residence time and enhance survival of the gene carrier and the DNA inside the harsh environment of the cytoplasm, which harbors several nucleases[29].

Nuclear import. Nuclear import of exogenous DNA from the cytosol is very inefficient and is considered as the predominant limiting step in gene delivery. Only small fractions (<0.1%) of pDNA delivered into the cytosol of cells using micro-injection will eventually reach the nucleus[30]. The size of the plasmid DNA prevents passive diffusion through the nuclear pore complexes (NPC) into the nucleus. Karyophylic proteins and also some viruses use the nuclear import machinery to be carried through the NPC into the nucleus. This import machinery consists of cytoplasmic karyopherins that recognize and bind specific amino-acid sequences in karyophilic proteins called nuclear localization sequences (NLS). They then dock onto the cytoplasmic side of the NPC, and the complex is then translocated into the nucleus. The nuclear import of DNA can be enhanced by using synthetic NLS peptides[31–33]. The success of NLS-mediated import of DNA is thought to be dependent on the size of the DNA constructs, but also on how the NLS peptide is attached to the DNA[28,34,35].

Controllable and sustained transgene expression. Besides delivery, control over transgene expression is another requirement for effective gene therapy. Currently used plasmid vectors in non-viral gene-delivery systems give transient and uncontrolled expression of the transgene. This is not a problem when the aim is to kill cells, as in cancer therapy. However, for other therapeutic applications that require a sustained and adaptable level of recombinant proteins in diseased cells, it would be desirable to have better control over the level and duration of gene expression. Gene regulatory systems have been developed that allow chemical control over transgene expression[36]. Particularly interesting for gene-therapy applications are systems in which transgene expression can be pharmacologically regulated using oral drug formulations[37].

Plasmid vectors normally used for gene delivery cannot replicate in human cells and are therefore lost upon degradation or during cell division. For this reason, transgene expression with regular plasmid vectors generally lasts for only 1–2 weeks at most. If persistent (months to years) transgene expression is desired, either self-replicating vectors or vectors that can stably and safely integrate into the genome of the host cells are needed[38].

Artificial viruses: a conceptual model

The major challenge of building artificial viruses is to unite all the required components mentioned above into a single nanoparticle without losing any of the functionalities of the individual components. An artificial virus should remain stable during its transport through the body and should be able to 'sense' its environment and disassemble in a controlled fashion once taken up by target cells. The controlled intracellular disassembly should eventually lead to the delivery of associated plasmid DNA into the cell's nucleus, where the transgene can be expressed.

Based on these requirements we propose a core-shell concept of an artificial virus (FIG. 3). The three main structural components of this artificial gene delivery system are the plasmid vector, engineered for optimal expression; the artificial virus core, consisting of pDNA, condensing agents and functional peptides; and finally the hydrophilic shell, exposing targeting ligands for cell-type-specific gene delivery.

TABLE 2 shows a selection of some of the work done on non-viral gene delivery systems that can be certified as artificial virus systems. Although the building blocks used to make the artificial viruses differ in each study, the concept is in principle the same: try to mimic viruses in delivering genetic material into specific host cells. We have constructed a non-viral gene-delivery system that shows similarity to enveloped viruses in its architecture. It consists of a core of pDNA condensed with cationic polymers and coated with a layer of anionic phospholipids. Antibodies that could specifically recognize cells expressing epithelial glycoprotein-2 (EGP2) were attached to this

envelope[16]. Similarly, Fahr *et al.* constructed an artificial virus consisting of a core of pDNA condensed with the cationic polymer polyethylenimine (PEI) and coated with the reconstituted envelopes of a retrovirus[39]. Specific cell targeting was achieved with RGD peptides that generate specificity towards endothelial cells. Although there is still room to improve the transfection efficiency of these systems, they clearly show that artificial viruses can be constructed from both synthetic and biological components, and can be based on the features and architecture of native viruses.

Artificial virus assembly: opposites attract. Assembly of the artificial viruses starts with the condensation of plasmid DNA using cationic materials. This electrostatically driven self-assembly has been extensively studied and described for binary systems (that is, negatively charged DNA with linear or branched polycations). It involves the formation of interchain electrostatic bonds that results in self-assembly of complexes. The process of complex formation is dependent on many factors such as pH, ionic strength and composition of the medium, temperature and concentration of DNA and cationic polymers[40]. Under well-chosen and controlled conditions, this self-assembly process can yield small, positively charged complexes. Similarly, well-defined complexes can be

Table 2 | **A selection of research groups that develop artificial virus-like gene-delivery systems**

Principal investigator	Location	Artificial virus composition	Developmental stage	(Dis)advantages	References
Y. Aoyama	Dept of synthetic chemistry and biological chemistry, Kyoto University, Japan	Glycoclusters: neutral glycocluster nanoparticles made of pDNA complexed with glycocluster amphiphiles	*In vitro* transfection studies	+ Small size + Targeting capacity – Intracellular fate unknown	59
J. P. Behr	Laboratoire de Chimie Genetique associe CNRS/ULP Strasbourg, France.	Multi-component: folate-targeted nanoparticles	*In vitro* transfection studies	+ Small size + Targeting capacity – Intracellular fate unknown	60,61
C. Coutelle	Gene Therapy Research Group, Imperial College London, United Kingdom	Multi-component: NLS sequences fused to the tetracycline repressor protein that bind the pDNA in combination with cationic lipids	*In vitro* transfection studies	+ Nuclear targeting – No cellular targeting	56
A. Fahr	Institute of Pharmaceutical Technology and Biopharmaceutics, Philipps University, Marburg, Germany	Multi-component: RGD-targeted reconstituted adenoviral envelopes around PEI complexed DNA	*In vitro* transfection studies	+ High transfection efficiency – Partial cellular targeting – Use of viral proteins	39
H. Harashima	Laboratory for Molecular Design of Pharmaceutics, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan	Multi-component: transferrin-targeted polyplexes containing fusogenic peptides	*In vitro* transfection studies	+ Targeting capacity + Cytosolic release of genes	62
W. E. Hennink	Dept. Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, The Netherlands	Multi-component: polyplexes coated with lipids to which monoclonal antibodies as targeting ligands have been attached	*In vitro* transfection studies	+ Specific targeting + Stability in serum – Low transfection efficiency	16
S.W. Kim	Center for Controlled Chemical Delivery,Dept. Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, USA	Multi-component: polyethyl-enimine polyplexes coated with polyethylene glycol and targeted with RGD-peptide motifs towards tumour endothelial cells	*In vitro* transfection studies	+ Specific targeting	63
L. W. Seymour	Dept Clinical Pharmacology, Oxford, United Kingdom	Peptides: reducible polypeptides containing histidine and lysine residues	*In vitro* transfection studies	+ Endosomal escape + Low cytotoxicity – No cellular targeting	41
A. Villaverde	Institut de Biotecnologia i de Biomedicina, Universitat Autonoma de Barcelona, Spain	Chimeric proteins: β-galactosidase proteins engineered to contain RGD targeting motifs, NLS sequences and DNA condensing sequences	*In vitro* trans-fection studies *In vivo* trans-fection studies	+ Multifunctional + Proven successful *in vivo* – Potentially immunogenic	64,65
E. Wagner	Department of Pharmacy, Ludwig-Maximilians-Universität Mu nchen, Munich, Germany	Multi-component: poly(lysine) condensed DNA coated with PEG that is shed at low pH	*In vitro* trans-fection data *In vivo* trans-fection data	+ Dynamic system – Good transfection efficiencies – No specific targeting	52
M.C. Woodle	Intradigm Corporation, Rockville MD, USA	Multi-component: polyethyl-eneimine polyplexes coated with polyethylene glycol and targeting withRGD-peptide motifs towards tumour endothelial cells	*In vitro* trans-fection data *In vivo* efficacy data	+ Tumour specific + Small size + Proven successful *in vivo*	19

Table 3 | **Overview of the functional components of an artificial virus**

Functional component	External trigger	Action	References
Targeting ligands	Receptor binding	Receptor-mediated endocytosis	20,21,51
Stealth polymers attached to carriers with an acid-labile bond	Drop in pH (endosomes)	De-shielding	52
pH-dependent or independent membrane-destabilizing peptides	De-shielding or drop in pH	Endosomal escape	23,24
Disulphide cross-linked compaction agents (peptides or polymers)	Change in redox potential	Disassembly	41,42,53,54
Dynein-binding peptides, proteins	Binding to dynein molecular motors	Transport through the cytoplasm towards the nucleus	28,55
NLS-peptides or NLS-containing proteins	Binding to importin, transportin	Nuclear import, classical and non-classical pathways	32,33,56
Promoter/enhancer regions in plasmid vector	Promoter recognition	Transgene expression	43,57,58

NLS, nuclear-localization sequence.

formed from more than two components using electrostatic forces to drive the self-assembly process. This would allow incorporation of multiple functional components in a single artificial virus particle. For example, plasmid DNA can be coated with functional peptides that assist in intracellular trafficking and nuclear import of the DNA (for example, dynein-binding peptides and NLS peptides). As these functional peptides need to remain associated with the DNA during the transport through the body and through the cell's interior, a strong interaction between the peptides and DNA is needed. This can be achieved by fusing the peptides to DNA-binding proteins such as protamines or histones. The partially coated DNA can then be condensed into small, positively charged nanoparticles using reducible cationic structures (either polymers or peptides that contain sulphydril groups)[41,42]. This provides stability to the core complex outside cells, but once inside the reducing environment of the cytosol the core will slowly start to disintegrate.

The core of the artificial virus can subsequently be coated with a hydrophilic layer of polymers that should form a steric barrier around the nanoparticles, which prevents opsonization and immune recognition of the nanoparticles inside the body.

The polymers can be covalently attached to the core of the artificial virus using acid-labile bonds to allow shedding of the polymer layer once exposed to the low-pH environment inside the endosomes. Targeting ligands that can bind internalizing receptors that are specific, or at least selective, for the target cell population can be attached to the distal ends of the polymeric shell to enable target cell binding.

Artificial virus disassembly. Similarly to natural viruses, artificial viruses should function dynamically and be able to react to changes in their surroundings. The encounter with target cells should trigger a cascade of events that ultimately results in the delivery of the plasmid DNA into the nucleus of target cells. These events include controlled disassembly of the nanoparticle and sequential activation or exposure of associated functional domains that assist in the intracellular transport of DNA towards the nucleus. As the artificial virus is not an intelligent device that can anticipate which functionality is needed at a given stage in the cell-entry process, the controlled disassembly and functional activation of the carrier should be induced by biological triggers, such as change in pH, redox potential or the presence of specific degrading enzymes (for example, lipases or endopeptidases). An overview of external triggers that can induce the activation or exposure of functional elements of the artificial virus for each step in the cell entry process is given in TABLE 3. The entire process starts with the specific binding of artificial viruses to receptors displayed on the surface of target cells. This receptor binding should induce receptor-mediated endocytosis of artificial virus particles. The low pH inside the lumen of endosomes can trigger slow release of the shell polymers that were attached to the artificial virus core with an acid-labile linker. This will subsequently expose pH-dependent, membrane-destabilizing peptides that cause disruption of the endosomal membrane and release of the entire core complex into the cytoplasm. The reduced redox potential inside the cytoplasm can be used to induce slow disintegration of the core complex, while at the same time exposed dynein-binding peptides dock the artificial virus core to molecular motors that transport the artificial virus core towards the nucleus. At the perinuclear space the core will further disintegrate into structures small enough to be transported through the nuclear pore complexes. This process is mediated by NLS sequences bound to the DNA. Once inside the nucleus the transcription machinery of the host cell is used to drive the expression of the transgene. Tailor-made plasmids can be used that provide optimal control over the level and duration of transgene expression[43].

Concluding remarks

The design of artificial viruses is a multi-disciplinary task that requires an in-depth understanding of the physico-chemical mechanisms that drive the assembly of such nanoparticles. In addition, the biological processes that underlie the cellular uptake and intracellular processing of these gene-delivery systems have to be well understood. At present, many examples of versatile, self-assembling nanoparticles for the delivery of DNA can be found in literature and this number is continuosly growing (TABLE 2). Nevertheless, the artificial virus concept as gene-delivery system is still in its infancy and needs further basic research to develop its full potential to meet the objective of efficient, targeted gene delivery and long-lasting gene expression *in vivo*.

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Competing interests statement
The authors declare no competing financial interests.

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