PERSPECTIVE

Adenovirus in a Synthetic Membrane Wrapper: An Example of Hybrid Vigor?

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ABSTRACT Nucleic acid delivery applications require the development of carrier systems that are effective, selective, and non-toxic. Many different viral and non-viral approaches, including the use of retroviruses, adenoviruses, liposomes, and dendrimers, have been investigated. Unfortunately, issues still remain with regard to the safety and efficiency of these delivery vehicles. In this Perspective, the challenges of designing a stable vector that is capable of effective gene therapy are highlighted. Progress in the area is also presented, including the work of Kostarelos and co-workers appearing in this issue of *ACS Nano*, in which they describe a novel delivery vehicle that consists of lipid envelopes encasing viral nanoparticles.

The use of nucleic acid-based therapeutics such as siRNA, pDNA, or gene constructs against systemic diseases such as cancer, infection, inflammatory disorders, and other life-threatening conditions is a treatment modality that promises substantially higher specificity and patient individualization than the conventional small-molecule drug therapies in current use. Unfortunately, this promise remains largely unrealized due to the absence of clinically acceptable systemic delivery vehicles that are capable of effectively and selectively delivering their nucleic acid cargo to target tissues. Recombinant viral vectors such as adenovirus (Ad) or adeno-associated virus (AAV) offer the highest *in vivo* efficiencies; however, they suffer from tropism issues (*i.e.*, an inability to target them to tissues other than those they have evolved to infect) and are rapidly cleared from the bloodstream by the adaptive immune response that develops after their first injection. In addition, there are major concerns surrounding the safe use and manufacture of viral vectors that have limited their clinical utility. Nonviral vectors such as lipoplex (*i.e.*, nucleic acid:cationic lipid complexes), polyplex (*i.e.*, nucleic acid: cationic polymer complexes), and cationic dendrimer carriers can display better acute toxicity and long-term safety profiles in some cases, but unfortunately, they all suffer from low efficacy relative to viral vectors. This is true even in the least challenging cargo delivery environment—cell culture—where the growth media can be stripped of nucleases and serum proteins that would otherwise decrease the physical and chemical stability of the vector and where the vector is often added at extremely high concentrations that are unattainable *in vivo*. Consequently, even though more than two decades have passed since the first report of lipofection,[1] there is still a compelling need for the development of new vector approaches to realize the enormous potential of this field.

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In this issue of *ACS Nano*, Kostarelos and co-workers[2] describe a hybrid approach to this problem that is intended to blend the positive features of both viral and non-viral carriers. "Virosomes"—liposomal vehicles having components of the viral receptor and fusion machinery incorporated within the membrane bilayer—and related systems have been known for some time; however, those studies focused primarily on their use for vaccine development. The advantages of the strategy that Kostarelos and co-workers use for the delivery of nucleic acid cargo are (1) control over the surface composition of the carrier to suppress immune recognition and rapid elimination of the hybrid Ad-liposomes from blood circulation *via* the reticuloendothelial system (RES); (2) production of a delivery vehicle, the pharmacokinetic properties of which are well-known, based on the clinically proven liposomal carrier platform; (3) self-assembly of the carrier surface coating to enable rapid formulation and scale-up while avoiding the potential complications of direct covalent viral capsid modification[3–5]

See the accompanying Article by Singh *et al.* on p 1040.

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Published online May 27, 2008.
10.1021/nn800279s CCC: $40.75

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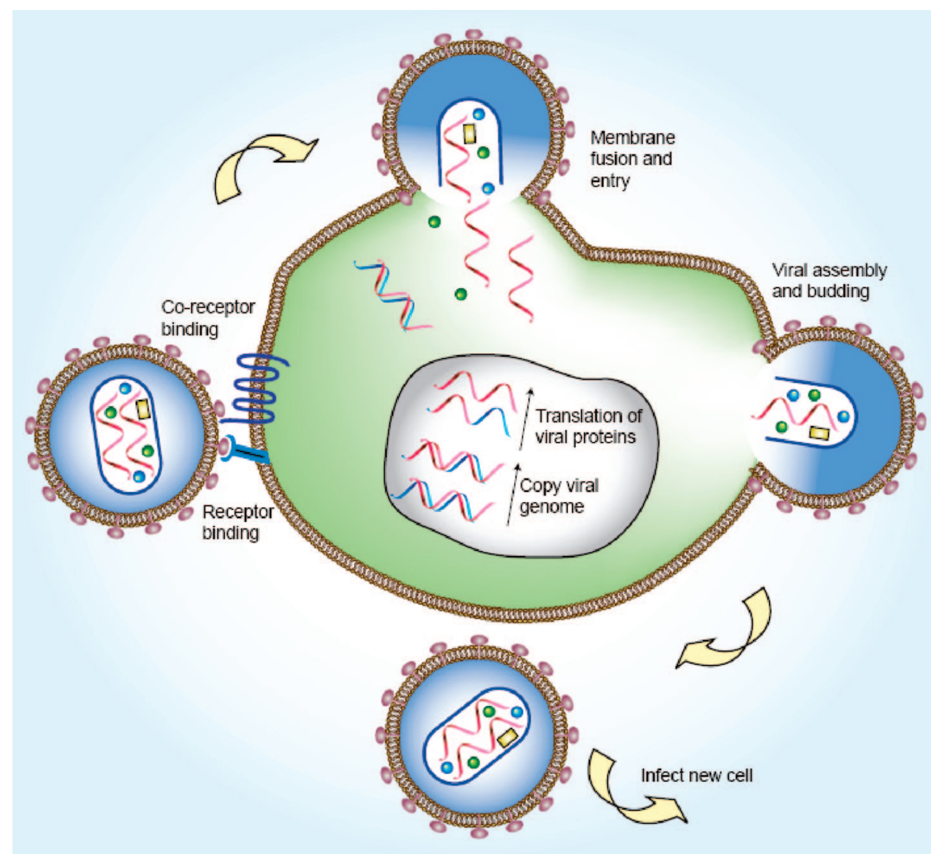

Figure 1. Schematic of the life cycle of a virus. Figure adapted from ref 10.

that may not go to completion; and (4) use of the efficient packaging and fusion machinery of the virus for passivation of the DNA cargo and its delivery across target cellular membrane barriers. The main disadvantages of this approach are a reduction in transfection efficiency and the need to either formulate the carrier with the well-known fusogenic lipid, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), or coadminister the hybrid Ad-liposomes with null Ad virus (*i.e.*, Ad virus that contains all functional viral components except the DNA cargo) to boost the DNA delivery performance of the carrier.

To put this contribution in perspective, it is worth noting the substantial challenges that must be overcome by any gene delivery agent that is intended for *in vivo* human therapeutic applications. Viruses provide the best model for how an efficient gene delivery machine should be designed. They have evolved to navigate through the body, select the target cell of choice, invade it, traffic to the desired subcellular compartment, escape the capsid vehicle and cross the membrane barrier of that compartment, co-opt the manufacturing capacity of the infected cell to produce multiple copies of the virion components, utilize membrane surfaces to scaffold the self-assembly of those components, and traffic to the cell surface where the viral particles escape without re-infecting the same cell (Figure 1). Even though most applications of nucleic acid therapeutics will only need to achieve the first five or six steps of this multistep process, there are no non-viral systems that are anywhere close to achieving this level of sophisticated functional integration in their design. Therefore, new carrier systems must be developed that incorporate as many of the factors listed below as possible to achieve improved efficacy.

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Safety. The carrier must evade immune surveillance and degrade into non-toxic, excretable byproducts.

Size. The carrier system must be greater than ~5 nm to avoid renal filtration and smaller than ~300 nm to enable efficient target cell internalization. It must also be reproducibly uniform in dimensions and surface chemistry to improve the chances for acceptance by the U.S. Food and Drug Administration (FDA). The primary role of the materials used for non-viral nucleic acid delivery is to condense the pDNA or siRNA drug into a small particle, relative to the persistence length of the uncomplexed nucleic acid cargo, so that it falls within the desired 5−300 nm size range. At this time, most carrier systems for systemic circulation are designed to be spherical; however, recent evidence suggests that there may be advantages provided by other carrier geometries.[6,7]

Cargo Passivation. A critical component of effective carrier system design is cargo protection. This is especially important for nucleic acid cargo that are susceptible toward nuclease degradation and immune recognition of their CpG motifs.[8] Most viruses achieve this by packaging their genomes within an icosahedrally symmetric capsid shell, whereas non-viral carrier systems typically utilize polyelectrolyte complexation or coating approaches.

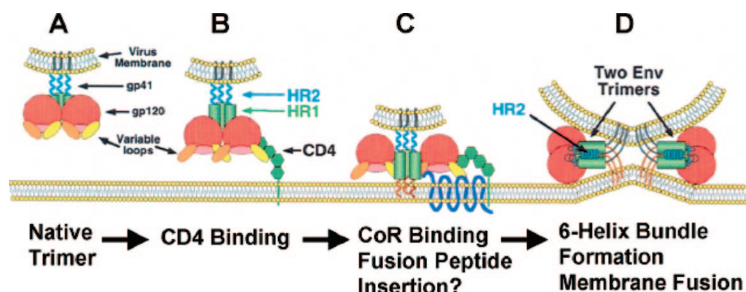

Figure 2. CD4- and CCR5-mediated entry of HIV into CD4$^+$ T-cells. After CD4 binding, gp120 undergoes a conformational change and exposes the co-receptor binding site on Env (step B). The triggered Env binds to the membrane co-receptor CCR5 (CoR), thus exposing a fusion peptide on the N-terminus of gp41 that inserts into the membrane of the target cell (step C). Co-R binding ultimately results in the formation of a six-helix bundle that bring the membranes into close proximity to induce local membrane curvature and fusion (step D).[10] Reproduced with permission from ref 10 and adapted from Moore, J.P.; Doms, R.W. *Proc. Natl. Acad. Sci U.S.A.* 2003, *100*, 10598-10602. Copyright 2008 World Scientific.

Steric Stabilization To Confer Long Circulation. Most carrier systems survive in the blood for relatively short periods, typically with less than 10% of the injected dose remaining after 4 h. This is due to RES activity, which is designed to scavenge foreign particles (*e.g.*, bacteria and viruses) and colloidal cellular degradation fragments from systemic circulation. Many viruses employ one or more membrane layers that are designed to mask the antigenicity of the viral coat proteins in an attempt to evade RES clearance. A common strategy used to extend the blood circulation time of non-viral carriers is the grafting of polyethylene glycol (PEG) onto the surface of the particle to exploit the so-called enhanced permeation and retention (EPR) effect.[9] This can greatly improve the pharmacokinetics of the carrier by masking the antigenicity of the particle surface, thereby slowing down the rate of opsonization (and hiding the antibodies that initially bind beneath a corona of PEG) that ultimately leads to RES clearance.

Tissue-Specific Targeting. Viruses typically employ two or more discrete targeting systems to enhance their target specificity. A well-known example is the HIV virus that requires the presence of CD4 receptors and CCR5 co-receptors (Figure 2).[10] Once the initial ligand–receptor molecular recognition event has occurred between the cell surface and the virus capsid shell, the redundancy of capsid ligand presentation on the viral particle surface creates multiple interactions with cell surface receptors. The i.v. net effect is a substantial increase in the binding affinity of the virus with the target cell due to this polyvalent interaction. Tissue specificity due to these combined effects has also been altered in the laboratory by mixing capsid proteins derived from different AAV serotypes to produce "cross-dressed" virions.[11]

Internalization. Polyvalent ligand–receptor interactions between the viral particles and their target cell can also trigger virus internalization *via* receptor clustering processes that induce clathrin coat formation and subsequent endocytotic uptake. Folate-mediated internalization is widely utilized in lipid-, polymer-, and dendrimer-based carrier systems due to the relative ease of the coupling chemistry, the high affinity of the folate receptor for folic acid, and the rapid internalization of folate conjugates into acidic endosomes.[12]

Trafficking. Viruses typically deliver their genomes within 30 min of internalization because they exploit microtubule networks to traffic to the nucleus. At least one non-viral system has utilized this mechanism to transport carrier-based xenocargo to the nucleus.[13]

Decomplexation. A key barrier to efficient nucleic acid delivery is the unpackaging of the cargo once it has entered the endosomal compartment. Viruses achieve this by disassembling their capsid shell upon endosomal acidification and

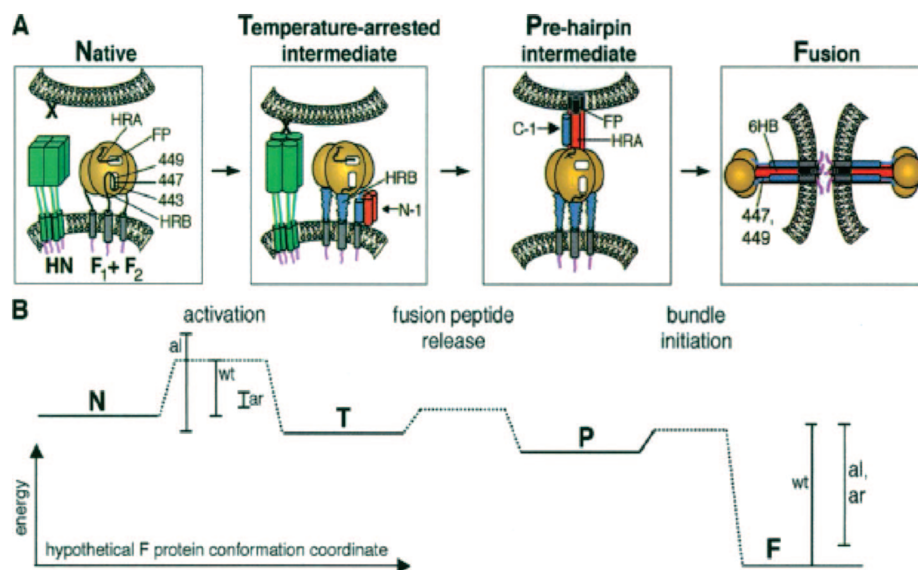

Figure 3. Model of paramyxovirus membrane fusion after ligand–receptor binding.[24] (A) The native structure is metastable and produces a pre-hairpin intermediate after (and independent of) HN ligand binding. Pre-hairpin intermediate formation reveals a cluster of fusion peptides that embed in the target cell membrane after assembly into a six-helical bundle. Rearrangement of the pre-hairpin intermediate into a hairpin structure that re-docks the fusion and transmembrane peptides (*i.e.*, analogous to the temperature-arrested intermediate) provides the energy needed for membrane curvature and fusion. (B) Biophysical experiments show a net loss in free energy that accompanies rearrangement of the system components. Image reproduced from ref 24. Copyright 2003 Rockefeller University Press under Creative Commons Attribution-Noncommercial-Share Alike 3.0 Unported license.

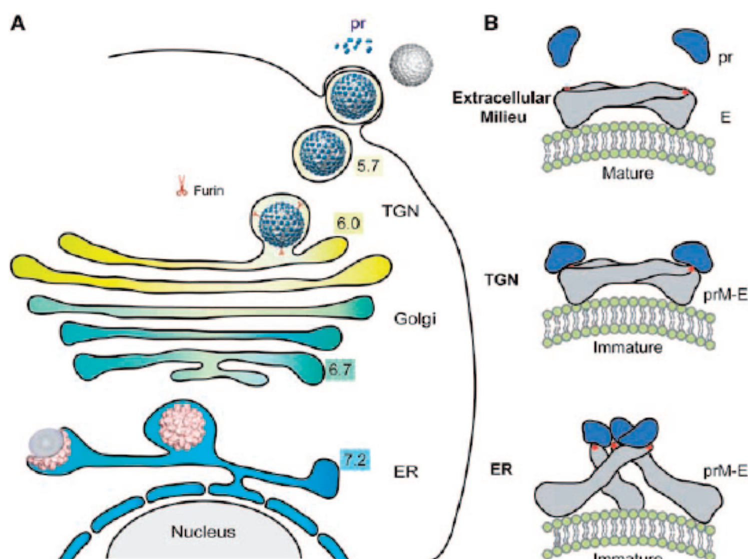

Figure 4. (A) Model of flavirus (*e.g.*, Dengue) maturation pathway.[26] (B) The capsid contacts are altered during viral maturation. Masking of the fusogenic peptides (highlighted in red) is conferred by the pr fragments. From ref 26. Reprinted with permission from AAAS.

transport of their genome across the endosome membrane (see below). Several different approaches have used non-viral carriers, including degradable lipids[14–19] and polymers[20–23] that are designed to release the nucleic acid cargo by converting the polycationic:polyanionic complex into cationic fragments that are more exchangeable than the intact complexes.

Endosomal Escape. Once they have gained entry into the infected cell *via* endocytosis, internalized viruses undergo structural transformations that are programmed to respond to the acidification process that naturally occurs within the endosomal compartment. These structural changes ultimately lead to fusion of the viral and endosomal membranes, with release of the viral genome into the cytoplasm of the infected cell. Figure 3 shows a cartoon of the multistep binding and fusion process for paramyxovirus and the attendant changes in relative free energy that occur with each step.[24] Cell-penetrating peptides, a family of arginine-rich peptides based on the HIV tat peptide that promotes transport across cellular membrane barriers, have been another popular choice for non-viral carrier systems. Kale and Torchilin[25] have recently reported the design of a liposome system that utilizes an acid-cleavable PEG to provide both steric stabilization properties and a masking effect of tat peptide that is buried beneath the PEG. Upon endosomal uptake, the PEG is removed from the liposome surface, thereby revealing the tat element that promotes endosomal membrane permeation and delivery of the pDNA cargo to the cytoplasm. New structural insights into the Dengue virus maturation and cellular escape process[26] show that this virus employs a similar type of masking mechanism to block interaction of membrane fusion peptides on the virus surface with the membranes of the exocytotic pathway, thereby preventing re-entry of the virus into the already-infected cell (Figure 4). In this case, the action of a peptidase leads to the cleavage of a viral surface protein; the proteolytic fragment conceals the fusogenic peptides as long as the virion is encapsulated within the export vesicle. Once the viral particle undergoes exocytosis, the protein fragment is then free to exchange off the virus surface, thereby revealing the fusogenic surface sequences of the mature virus.

Endosomal escape has been shown in many different cellular trafficking studies of non-viral carriers to be the most important factor in determining the efficacy of the internalized nucleic acid cargo. The mode of endosomal escape by non-viral carriers is often attributed to one of two different mechanisms—endosomolysis (*via* the so-called "proton sponge" effect or, in some cases, by detergent solubilization) and direct membrane−membrane fusion. It should be noted that Kostarelos and co-workers utilized null Ad virus in a post-treatment approach to effect endosomal escape of the nucleic acid cargo by exploiting the intrinsic fusogenicity of Ad virus with the lipid-enveloped Ad viruses whose fusogenic capacity was blocked when they added their synthetic lipid bilayer membrane coating.[2]

Nuclear Localization. Nuclear localization sequences (NLS) are employed by viruses to accelerate the transport of the viral genome to the nucleus, where it becomes activated. Many non-viral carrier systems have utilized NLS; however, these typically display little improvement in nucleic acid efficacy

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in the absence of strategies that also take advantage of internalizing targeting ligands and/or endosomal decomplexation mechanisms.

Effectiveness of Nucleic Acid Cargo. Stable expression of the viral genome is achieved by its integration into the host cell genomic DNA. Design of pDNA for enhancing transient expression or siRNA for improved knockdown levels are the primary strategies employed in non-viral nucleic acid delivery systems. This lower level of function for non-viral carriers is related to both the difficulty of achieving genomic integration and the safety issues related to the reliability of genomic locus insertion and the degree of gene regulation that can be achieved with non-viral systems.

Manufacturability. Any effective nucleic acid carrier system will ultimately need to address the issues of safe manufacture in large volumes, with reproducible production and shelf stability, and at low cost before gaining clinical acceptance and FDA regulatory approval.

Due to the multiplicity of factors required for highly effective nucleic acid delivery, the performance of non-viral vectors is typically low, since very few have been designed to be responsive to more than one or two steps in this multistep process (*i.e.*, most address either complexation, cargo passivation, *in vitro* efficiency, or toxicity). Although a functional relationship between nucleic acid complex structure and performance has been recently described for pDNA lipoplexes,[27] the "readout" of the carrier function is the product of multiple steps, many of which are mechanistically ill-defined and occur in places and on time scales within the target cell that are unknown, making it challenging to know how to go about system optimization.

Opportunities for Nanoscale Nucleic Acid Carrier Systems. The need to develop effective nucleic acid delivery agents continues to grow, particularly now that siRNA is recognized as an increasingly important cargo for therapeutic applications. This opportunity is accompanied by a growing list of "nanomaterial *du jour*" appearing in the literature, many of which are claimed to be relevant to drug or nucleic acid delivery because of fashion, funding, and ease of fabrication. Unfortunately, the overwhelming majority of these materials and approaches fail to address the real issues that face the non-viral vector field. *The most pressing needs for the development of efficient non-viral carrier systems are improvements in targeting efficiency and selectivity.* These rank as most important because they are the first step in the nucleic acid delivery process; any inefficiencies in this step propagate throughout the entire system performance. Increased understanding of internalization mechanisms, as well as the factors that affect trafficking patterns and the time scales of those processes, is also needed so that functional elements that respond within the same kinetic window can be built into the delivery vehicle strategy. Many non-viral nucleic acid delivery systems are developed without factoring these criteria into their design. *Too often the focus is on the structure of the material rather than on the kinetic regimes and environmental milieu that the carrier systems will encounter as they travel through the circulatory system and the endocytic pathway. These efforts are typically unsuccessful because they fail to recognize that time and place are just as important as structure in biological processes.* Consequently, this field will advance significantly only when multifunctional carriers are developed that are responsive to several key steps in the pathway. Once these factors are more fully addressed by improved vehicle design, we can anticipate that our efforts will lead to non-viral systems, whose performance is comparable to the efficacy of viral systems. Kostarelos and co-workers have provided some clues as to how these challenges can be met.[2] Indeed, the impressive performance they report in tumor spheroids, where even the native virion particles have a difficult time transfecting cells that are deeply embedded in the tumor mass compared to the lipid-enveloped viruses, suggests that the use of multifunctional carriers comprised of a viral/non-viral hybrid may be a promising new approach for efficient nucleic acid delivery.

The most pressing needs for the development of efficient non-viral carrier systems are improvements in targeting efficiency and selectivity.

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