European Journal of Pharmaceutics and Biopharmaceutics 71 (2009) 475–483

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Review article

Recent development of nonviral gene delivery systems with virus-like structures and mechanisms

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ARTICLE INFO

Article history:
Received 25 February 2008
Accepted in revised form 2 September 2008
Available online 14 October 2008

Keywords:
Nonviral gene delivery
Gene therapy
Biocompatibility
Nanocarrier
Polyplex micelle

ABSTRACT

The concept of gene therapy includes not only the addition of normal genes to genetically deficient cells, but also the use of transgenes encoding several peptides that function to enhance the capacity of normal cells or to regulate cell differentiation. The application of gene therapy has been widely considered for various diseases, as well as for the field of tissue engineering. To overcome the problems with viral vectors, a broad range of nonviral systems for gene delivery have been developed, including systems composed of cationic lipids (lipoplexes) and cationic polymers (polyplexes). However, most of these systems are still much less efficient than viral vectors, especially for *in vivo* gene delivery. Paradoxically, to achieve a maximum transgene expression in the targeted cells, there is no question that natural viruses are the most effective nanocarriers. In this article, we highlight the approaches currently being taken to improve nonviral gene delivery systems so that they better replicate the typical structures and mechanisms of viruses, such as DNA (RNA) condensation in the core, surrounding structures with targeting molecules for specific receptors, as well as the toxic and immunogenic problems which should be avoided, with the ultimate goal of bringing these systems into a clinical setting.

1. Introduction

As our knowledge of the molecular background of various diseases continues to grow, gene therapy is becoming an increasingly attractive possibility in a wide range of diseases, as well as one of the great scientific challenges in modern medicine. The concept of gene therapy includes not only the addition of normal genes to genetically deficient cells, but also the use of transgenes encoding several peptides that function to enhance the capacity of normal cells or to regulate cell differentiation. The advantages of using transgenes for therapeutic purposes compared with delivery of exogenous proteins or bioactive molecules are (1) the transgene can express bioactive factors in the native form at the targeted site [1]; (2) the sustained synthesis of proteins from the transgene can facilitate synchronization between the kinetics of signaling receptor expression and bioactive factor availability [2]; and (3) transgenes are more flexible in terms of their potential applications. The sequence-specific inhibition of gene expression using antisense DNA and siRNA (and its expression vector) is also considered to be a type of gene therapy. Gene therapy has widely been considered not only for lethal diseases such as congenital hereditary disorders, end-stage malignant tumors, and severe infectious diseases, but also cardiovascular diseases [3], age-related degenerative diseases [4], wound healing [5] and tissue engineering [1,6].

Despite this great promise, however, there have been few successful outcomes of gene therapy so far. Early clinical trials using recombinant viral vectors have reported significant problems, such as short-term transgene expression, an inability to persist in host cells and toxicity. In 1999, direct injection of an adenovirus vector into the hepatic artery caused the death of a patient [7]. Analysis of the inflammatory cytokine profile indicated that the vector had caused systemic inflammatory response syndrome, resulting in intravascular coagulation, acute respiratory disorder and multiorgan failure [8,9]. Viral vectors were reported to be somewhat more successful in the treatment of children suffering from the fatal form of X-linked severe combined immunodeficiency disease (SCID-X1 syndrome) in 2000 [10]. The immune systems of these children were rendered functional by stem cell gene therapy using a retrovirus vector, but unfortunately, five patients so far developed a leukemia-type disease owing to insertional mutagenesis [11,12].

These results highlight the need for nonviral systems for gene delivery. In response to the observed problems with viral vectors, a broad range of nonviral systems for gene delivery have been developed, including systems composed of cationic lipids (lipoplexes) and cationic polymers (polyplexes). Such nonviral gene

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doi:10.1016/j.ejpb.2008.09.019

carriers may also trigger an inflammatory response but are not likely to induce specific recognition, making the carriers less hazardous in terms of antigen-specific immune response. However, most carriers are still much less efficient than viral vectors, especially for *in vivo* gene delivery. Paradoxically, to achieve a maximum transgene expression in the targeted cells, it is clear that the natural viruses are the most effective nanocarriers. For development of improved nonviral carriers for human gene therapy, an improved understanding of the mechanisms in natural viruses and its application to the molecular design of nonviral carriers would be beneficial.

In this article, we highlight the approaches currently being taken to improve nonviral gene delivery systems so that they better replicate the typical structures and mechanisms of viruses, such as DNA (RNA) condensation in the core, surrounding structure with targeting molecules for specific receptors, and the toxic and immunogenic issues which should be avoided. This review includes a discussion of our own recent efforts to improve nonviral gene delivery using polyplex micelles composed of poly(ethyleneglycol) (PEG)-block-polycations.

2. DNA condensation

Although there are many structural variations among virus species, most viruses are characterized by the presence of condensed DNA (or RNA) in the core and a multitiered structure surrounding the core. Apparently, the condensation of DNA – with one or more shells of protein wrapped around the DNA in an often helical or icosahedral structure called the capsid – is beneficial to protect the DNA or RNA genome from physical or enzymatic degradation [13,14]. In the case of enveloped viruses, there is also a lipid bilayer membrane that serves to protect the genome and the capsid itself.

Recently, the mechanisms underlying DNA packaging into the capsid have begun to be clarified [15,16]. Bacteriophages, herpes viruses and other large double-stranded DNA viruses possess sophisticated molecular mechanisms that pump DNA into preassembled procapsids. The packaging is initiated by recognition and cleavage of the specific *pac* sequence (*pac* cleavage), which generates the first DNA end to be encapsidated. A sequence-independent cleavage (heedful cleavage) terminates packaging, creating a new starting point for another round of packaging. These 'headful packaging' processes are ATP-dependent and, surprisingly, the internal capsid pressure exceeds, by 10-fold, that of bottled champagne. The structure of the tight dsDNA spooling has been shown to activate the switch that signals the headful chromosome packing density to the particle exterior [16]. Although the detailed mechanism of the packaging of circular DNA is unknown, the viruses clearly have active and sophisticated mechanisms to condense the DNA inside the core.

In contrast, the DNA condensation in nonviral gene carriers is achieved via electrostatic interactions between the anionic phosphate groups of DNA and the cationic molecules of the carriers [17]. Generally, polymer-based gene carriers possess positively charged groups that are known to condense DNA coils into 10^{-3}–10^{-4} of the original volume by forming a polyion complex (PIC) [18]. The condensed state of DNA significantly increases the tolerance from degradation by DNases existing in the body [19]. However, when a single cationic homopolymer is used, the condensation of DNA is likely to be a random process dependent on the order and rate of mixing DNA and polymer solutions, causing considerable polydispersity of the carriers. Overstabilization of DNA may also occur when there is an excess of cationic polymers, which inhibit the smooth release of DNA through an interexchange reaction with counter polyanions inside the cells [17,20,21].

In order to regulate the status on DNA condensation, the use of cationic block copolymers with a hydrophilic segment is an interesting possibility. Due to the formation of polyplex micelles in which the PIC is surrounded by a hydrophilic poly(ethyleneglycol) (PEG) palisade, a water-soluble structure with condensed DNA can be obtained with narrow dispersity [22,23]. Using polyplex micelles of PEG-Polylysine (PLL) block copolymers and plasmid DNA, the condensation behavior of DNA was investigated by adding an S1 nuclease (single-strand specific cleavage enzyme) that cleaves the looped DNA strand, with the fragmentation of DNA expected to reflect the destabilization of DNA double strands. When the micelles were formed in a stoichiometric ratio of nitrogen in cationic segment to DNA phosphates, a surprising digestion behavior by the S1 nuclease was observed. The condensed DNA was separated into seven distinct fragments, which were 10/12, 9/12, 8/12, 6/12, 4/12, 3/12, and 2/12 the length of the original plasmid DNA, respectively [24,25] (Fig. 1). Moreover, the ordered fragmentation occurred in the full series of plasmid DNAs, which ranged in size from 2200 to 12,000 base pairs, suggesting that it was related to the inherent propensity of plasmid DNA. Note that no such ordered fragmentation was observed using polyplexes formed of PLL homopolymer, but the DNA was degraded in a nonspecific manner. These results suggest that the condensation of DNA in the polyplex micelles occurred under a regulated mechanism of DNA folding.

The important point is that the high-order structure of DNA may be strongly related to its biological activity. When the condensation is complete and the DNA molecules are fully packed, there is complete inhibition of enzymatic action, such as transcription [26]. However, when the folded structure is a swollen globule, enzymes can access DNA molecules. Indeed, based on studies of chromatin structure, it has been established that tightly packed and loosened parts coexist except during mitosis, presumably reflecting the on/off switching activation of a large number of genes [27]. It is reasonable to speculate that regulated condensation of DNA inside the carriers is crucial to smooth intracellular processes for efficient transcription. Our preliminary investigations have revealed that, just by changing the condensation state of DNA in the polyplex micelles, the transcription activity was significantly affected in both a cell-free examination and *in vivo* experiments. In nonviral systems, it may not be possible to actively package DNA in the manner of natural viruses in the near future. Nevertheless, studies on the regulation of DNA condensation should receive greater emphasis over the long-term in order to design sophisticated molecular structures for the nonviral carriers.

3. Poly(ethyleneglycol) (PEG)

Natural viruses have a multitiered structure surrounding the core that serves to protect the genome and operate as a 'transport vesicle' during cell-to-cell transmission. In nonviral systems, poly(ethyleneglycol) (PEG) has often been used for a similar purpose to protect polyplex and lipoplex-type gene carriers. Attachment of hydrophilic polymers such as PEG to the surface of liposomes, to create what are known as stealth liposomes [28], shields them from undesired binding activity in the blood, resulting in prolonged circulation times [29–31]. Many researchers have applied the same concept to shield targeted DNA polyplexes, and several strategies have been developed for the attachment of PEG to polyplexes [32]. The hydrophilic polymer was covalently coupled to the DNA-binding polycation either before polyplex formation (pre-PEGylation) [33,34] or after the polyplex formation (post-PEGylation) [35,36]. Self-organization into micellar structures using amphiphilic block copolymers composed of PEG and cationic segment has also been reported [22,23,36–39]. Shielding by PEG increased solubility, provided stability, reduced toxicity and extended the circulation time of polyplexes in the blood.

Although the PEG shield may increase systemic delivery to the target cells, it reduces gene expression activity within the target