Expert Opinion

Polymers for viral gene delivery

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Background: The development of viral vectors capable of providing efficient gene transfer in diseased tissues without causing any pathogenic effects is pivotal for overcoming the many challenges facing gene therapy. *Objective*: Immune responses against viral vectors, inadequate gene expression and inefficient targeting to specific cells *in vivo* are some of the major problems limiting the clinical utility of viral gene therapy. *Methods*: This review will focus on recent progress in strategic polymer-based modifications to improve the performance and biocompatibility of a variety of viral vectors. We will discuss the preclinical development of four approaches involving injectable polymers, polyelectrolytes, polymer microspheres and polymer–virus conjugates. *Results/conclusion*: Much progress has been made in creating 'hybrid' gene delivery vectors that combine the strengths of polymers and viruses. With further optimization, these hybrid vectors, which may be safer and more effective, are likely to succeed in clinical applications.

Keywords: gene delivery, gene therapy, polymers, viral vectors

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1. Introduction

The ability of viruses to recombine during growth makes them a potential target for use in delivering functional genes to restore protein synthesis in defective cells of genetically inherited or acquired diseases. Most common viral vectors currently being developed for gene delivery are based on retrovirus and adenovirus (Ad). Other viral vectors of interest include adeno-associated virus and herpes simplex virus. While retrovirus and adeno-associated virus can integrate their genetic material into the genome of a host cell following infection, adenovirus and herpes simplex virus type 1 (HSV-1) are non-integrating vectors that can only provide transient gene expression [1]. Inherited conditions requiring permanent gene replacement and gene expression generally require the use of retrovirus for integration of a therapeutic gene into the genome of target cells. For other medical applications, transient gene expression provided by adenoviral vectors may be sufficient.

Regardless of vector, a fundamental requirement in the development of viral gene delivery vehicles is the generation of replication-defective viruses. Several recombinant viruses that meet this criterion have been produced for use in gene therapy studies, although their clinical utility has yet to be realized. Development of viral vectors for clinical applications is not without issues, limitations and/or risks. Toxicity due to lack of cellular specificity or pre-existing immunity against viral vectors often prohibits the repeated administration necessary to achieve therapeutic effects. Safety remains an issue associated with the use of viral vectors, as they can potentially induce mutagenesis and carcinogenesis. In some instances, the generation of a replication-competent virus has been observed [2]. This is an added safety concern that is being addressed through the development of assay capable of detecting replication-competent Ad [3].

Several barriers must be overcome to achieve successful viral gene delivery. Substantial efforts have focused on strategies aimed to prolong transgene expression and improve targeting ability, as well as safety of the vectors. Progress in recent

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years toward the development of non-viral drug delivery systems using polymers and lipids has led to the design of 'hybrid' vectors that are partly viral and partly non-viral. In these hybridized vectors, viral particles are usually chemically modified through covalent attachment of polymers, encapsulation, direct conjugation, or other methods of alterations. As such, they are endowed with the ability to evade the surveillance of their host immune system and other mechanisms of clearance. Encapsulation of viruses also allows for controlled release of the vectors and prolonged transgene expression. Strategic introduction of ligands that recognize specific receptors expressed abundantly and uniquely on target cells is reported to be an effective approach for developing gene delivery vectors capable of targeting diseased tissue with minimum toxicity and reduced off-target expression in healthy tissues.

This article provides an overview on recent advances in the development of polymer–virus hybrid vectors with improved infectivity, targeting ability and safety profiles. A brief summary of commonly used viral vectors and polymers will be given, followed by a detailed discussion of novel polymer-based modifications of viral vectors, with emphasis on the strategies employed and the *in vitro* and *in vivo* effectiveness of each method.

2. Commonly used viral vectors

2.1 Adenovirus

The interest in developing Ad vectors as gene delivery vehicles arises from the ability of Ad vectors to transduce efficiently both in dividing and non-dividing cells, their broad tissue tropisms and low pathogenicity.

Recombinant Ad is frequently used as a model vector for gene therapy studies. They can be produced in high titers, with large capacity (up to 36 kb) for insertion of therapeutic DNA, and can be modified with ease in the laboratory. Although more than 50 human serotypes from six different groups (A – F) have been isolated, vectors derived from Ad serotype 2, and 5 of group C, are most intensively used in studies. Their host range is known to resemble that of the wild-type virus. All Ad serotypes except group B generally use the coxsackie- and adenovirus-receptor (CAR) to initiate cell binding [4]. Subsequent interaction between an RGD-motif at the penton base protein with cell integrins ($\alpha_v\beta_1$, $\alpha_v\beta_3$, or $\alpha_v\beta_5$) allows for virus entry through clathrin-mediated endocytosis [5]. Initial virus attachment of some Ad serotypes to a target cell has been recently suggested to involve receptors other than CAR [6,7]. The broad host range of Ad serotypes allows for altering the use of cellular tropism to achieve tissue-specific transduction [1].

Recombinant adenoviruses are constructed by deleting the early gene essential for virus replication and replacing it with a therapeutic gene. The expression of a gene transferred by an adenovirus vector is efficient but transient. Commonly used Ad2 and Ad5 vectors are highly immunogenic, so they cannot be administered repeatedly [8]. Recent development of Ad vectors with serotypes 11, 35 and 49, which do not have pre-existing immunity in humans, have been achieved [9-11]. The inherent immunogenicity of the immunogenic Ad vectors, however, has been exploited as a desirable feature for vaccine development [12,13].

The first replication-deficient Ad vectors constructed had the E1 and sometimes E3 gene region replaced by the introduction of a foreign gene, and are referred to as first generation Ad (FG-Ad). FG-Ad provide effective but short duration of transgene expression, due to their inherent ability to elicit immune responses, which leads to rapid clearance of vector-transduced cells [14]. Second generation Ad vectors (SG-Ad) are generated with the additional removal of E2 and/or E4 region to yield vectors with increased packaging capacity, reduced inflammatory response and extended duration of transgene expression [15]. Construction of a highly attenuated helper-dependent adenovirus (HD-Ad), also referred to as last generation or gutless adenovirus, further improves both the problems of immunogenicity and packaging capacity. HD-Ad is constructed with all viral genes removed except for the ITRs and the packaging signal (ψ) [16,17]. HD-Ad can accommodate up to 36 kb of non-viral DNA and exhibit long-term, high-level transgene expression *in vivo*. Nevertheless, host responses against HD-Ad capsid protein still persist in preventing successful gene expression upon re-administration [18]. Continuous efforts are therefore needed to develop safer and more efficient recombinant Ad vectors for use in a wide variety of gene therapy applications such as the treatment of cancer, cardiovascular disease or the formulation of a vaccine for HIV-1.

2.2 Retrovirus

Retroviruses, the most common vectors studied to date, consist of a large family of enveloped RNA viruses. The viral envelope glycoprotein determines the host range of retroviral particles and mediates viral interaction with receptors on target cells [1]. Due to the limited cellular tropism of the viral natural envelope, retroviral transduction is generally poor. Incorporation of related or different viral *env* glycoproteins or pseudotyping allows for improvement of transduction efficiency [19,20]. An attractive feature of retroviruses is their ability to integrate the therapeutic gene into the genome of target cells and establish a long-lasting effect. Several types of retroviruses have been developed for gene therapy, namely the oncoretroviruses, lentiviruses including HIV-1, and spumaviruses also known as foamy viruses. The murine Moloney-based oncoretrovirus vectors are most commonly used since they have no known associated human diseases [21]. Lentivirus vectors are used increasingly, both *in vitro* and in animal studies, due to their ability to infect non-dividing cells. Despite extremely high transduction efficiency, it is often challenging to produce retroviruses in large quantities with consistent quality. Integration with the

host genome can also be a serious safety concern for retroviruses. Due to at least part of these challenges, retroviruses are mostly used *ex vivo*.

2.3 Adeno-associated virus

Adeno-associated viruses (AAV) are members of the dependovirus and its subfamily of the parvoviridae. They are non-pathogenic, non-replicating DNA viruses. Of more than eight available serotypes, AAV serotype 2 (AAV-2) is commonly used as it shares the natural tropism of the wild-type virus, displays long-term transgene expression and is minimally immunogenic [22]. AAV-2 has limited use for vascular and endothelial cells due to lack of cell tropism and extracellular matrix sequestering of vectors, respectively. AAV mainly use heparin sulfate proteoglycans receptors to initiate cells binding and co-receptors integrin $\alpha_v\beta_5$ and fibroblast growth factor to facilitate internalization [1]. Recombinant AAV (rAAV) have the ability to transduce a wide range of tissues [22,23] and provide long-term expression [24-26]. Limitations of AAV include small packaging capacity, pre-existing AAV-specific antibody and strong immune response generated against viral capsid [27]. AAV have been widely employed for cardiovascular gene therapy [28] and for treatment of other conditions such as cancer, Parkinson's disease, cystic fibrosis, etc. [29]. Recent work also supports the use of AAV in gene delivery to the nervous system [30].

2.4 Limitations of viral gene delivery

While recombinant viruses offer many advantages as gene delivery vectors, several limitations have precluded their use in clinical settings. Obstacles presented in systemic delivery of vectors include the lack of cell/tissue specificity, rapid clearance of viral vectors from the body and physiological barriers to virus transport to target cells [31]. In some cell types, viral vectors fail to transfer the high level of genes required to achieve therapeutic effects. For example, Ad vectors have demonstrated a low level of transgene expression in vascular endothelium, smooth muscle and airway epithelium cells due to their resistance to Ad infection [32,33]. The inability of some retroviruses to transduce target cells efficiently has been attributed to the slow rate of virus binding to cells [34,35]. Site-specific delivery of vectors, in contrast, could also pose a problem as vectors can diffuse freely *in vivo* following administration, resulting in undesirable side effects [36]. In addition to the well-appreciated risk of uncontrolled genomic insertion by viral vectors that integrate transgenes with the host genome, an equally adverse factor in gene therapy is the pre-existing immune responses or acquired immunity in the human population against viral vectors [37]. In both cases, neutralizing antibodies quickly clear viral vectors from circulation, reducing the efficacy of the vectors and rendering repeated administration impossible. In extreme cases, of course, acute immune reactions toward viral vectors may lead to destructive consequences, and even the death of patients [38].

3. Polymers for delivering viral vectors: an overview

Pharmaceutical research to date has identified many biodegradable and biocompatible polymers for controlled delivery of a wide variety of therapeutic modalities, including small molecular drugs, peptides, proteins, nucleic acids (DNA, RNA) and even cells. Their use has since been effectively extended for the development of hybrid vectors in gene delivery to overcome the drawbacks of viral vectors previously mentioned. Within such hybrid vectors, the role of the polymer is passive, that is it primarily facilitates the delivery of viruses, whereas the active gene transfer function is carried out by the viruses. Other types of hybrid vectors of which the viral component is only facilitative, such as the Ad/polyethylenimine/DNA complexes [39], are beyond the scope of this review. Compared to other non-viral drug carriers such as lipids, polymers have distinct advantages. Being a macromolecule consisting of long molecular chains of various structures, polymers offer multiple active and reactive sites which enable cooperative interactions with the cargo, such as a drug or a virus, and as such can provide more effective protection and functionalization than small molecules. On the other hand, it is often desirable to control the release of cargo into specific physiological environments; polymers can be engineered chemically to be biodegradable, thus providing means to achieve such release. They can also be readily processed into physical forms of multiple length scales, such as macroscopic implants, microparticles and even nanoparticles to accommodate different therapeutic needs. Furthermore, they have a long and successful history of use in surgery and as implants and have established excellent safety records as approved products for human use, making them attractive materials to explore for assisting viral gene delivery.

In this article, the following four major categories of polymer-based approaches for viral gene delivery are discussed. The essence of the approaches, along with the benefits and limitations, are summarized in **Table 1** and illustrated in **Figure 1**. These include i) polymer matrix encapsulation: water soluble, biocompatible natural polymers such as collagen and recombinant silk and elastin-like proteins (SELPs), as well as synthetic polymers such as the poloxamers, are used to entrap viral vectors that can be delivered into the body through either surgical implantation or minimally invasive injection (**Figure 1A**). Viral vectors are thus protected and can be released slowly through diffusion and matrix erosion; ii) polyelectrolyte complexation: polycations such as polylysine, polybrene and polyethylenimine form polyelectrolyte complexes with negatively charged viral vectors (**Figure 1B**), sometimes in combination with additional polyanions (**Figure 1C**), resulting in viral aggregation. Polycations also neutralize negatively charged cell surface, enabling better gene transfer; iii) polymer microencapsulation: hydrophobic, biodegradable polyesters such as poly(lactic-*co*-glycolic acid)

Table 1. Summary of polymer-based approaches for viral gene therapy – main functions, benefits, limitations and selected examples of polymer systems discussed in this article.

Approaches	Main functions	Benefits	Limitations	Representative polymers	Selected ref.
Injectable polymer matrices	Macroscopic hydrogels formed *in situ* that trap viruses inside	Protects virus from damage, reduces immune rejection, prolonged release, local minimal invasive delivery, avoids viral dissemination	Difficult to control the rate of release *in vivo*	Collagen Alginate Pluronics (Poloxamer) Silk and elastin-like proteins Photocurable gelatin	[40-45] [46] [47-51] [52] [53]
Polyelectrolyte complexes	Binds and aggregates viral particles through electrostatic interactions	Protects virus from damage, enhances transfection by promoting cell entry, used mostly for *ex vivo* gene transfer	Difficult for *in vivo* and systemic delivery, structure of polymer–virus complex not well-defined	Polylysine, polybrene, polyethylenimine PEG-polylysine Cationic gelatin	[35,54,59-64] [65] [68,69]
Polymer microspheres	Traps viruses in microspheres formed through phase separation or cross-linking	Protects virus from damage, reduces immune rejection, prolonged release, local minimal invasive delivery, preferential uptake by phagocytic cells	Difficult to control the rate of release *in vivo*, viral activity lost during formulation, low efficiency of loading	PLGA PLGA/polylysine Alginate/Ca^{2+}/polylysine Gelatin/alginate Chitosan/bile salt Silica	[71-75,77,78] [76] [79,80] [82] [83] [84]
Polymer–virus conjugates	Hydrophilic polymers attach to individual viral particles covalently	Protects virus from damage, reduces immune rejection, systemic delivery, tumor targeting via the EPR effect, attaching molecular ligands for cell specific targeting	Difficult to achieve site-specific conjugation, viral activity lost post-conjugation	PEG (non-targeted) PEG (targeted) pHPMA (non-targeted) pHPMA (targeted)	[86-91,94-96,98-101] [92,93,106,107] [110] [111-115]

EPR: Enhanced permeability and retention; PEG: Polyethylene glycol; PLGA: Poly(lactic-*co*-glycolic acid); pHPMA: Poly[*N*-(2-hydroxypropyl)methacrylamide].

form microspheres to entrap viruses (**D**). Protected viruses can then be injected and released upon polymer degradation; and iv) polymer conjugation: linear water soluble polymers such as polyethylene glycol and poly(*N*-(2-hydroxypropyl)-methacrylamide) can be conjugated chemically to the viral particle surface either at one end of a polymer chain (**E**) or through multiple points of attachment via the polymer side-chains (**F**). These polymers can be further functionalized with cell-specific ligands to achieve targeting to specific cells and tissues (**G**,**H**). Polymer–virus conjugates have shown reduced nonspecific binding and cellular uptake *in vivo*, prolonged half-life, reduced immunogenicity and, in some cases, they can be retargeted to cells and tissues of interest. The chemical structures of some of the most studied polymers for viral gene delivery are shown in **Figure 2**.

4. Injectable polymer matrices

The use of polymer matrices, primarily hydrogels, as vehicles for delivering viral vectors locally to diseased cells has been explored as a strategy to reduce the spread of vectors to organs other than the target, to provide a sustained supply of viral vectors and sustained gene transfection and to minimize potential immune reactions against the vectors. Although viral vectors entrapped in polymer matrices can be surgically implanted into the patients, it is much more desirable clinically to use injectable polymers that can be delivered through minimally invasive procedures. Application of such hybrid polymer/virus systems has mainly been applied to cancer gene therapy and tissue repair and regeneration.

Most of the injectable polymer matrices for viral gene delivery consist of water soluble biocompatible polymers, including components of the extracellular matrix. Collagen type I is a widely used biomaterial for delivery of viral vectors *in vivo* because of its compatibility with tissues and cells. Typically, viral particles are suspended in (1 ~ 3%) collagen aqueous solution at 4°C at slightly acidic pH where collagen remains soluble. To initiate gelation, the virus/collagen mixture is brought to neutral pH and 37°C. The irreversible hydrogel formation of collagen in water due to a change in temperature offers a very convenient way

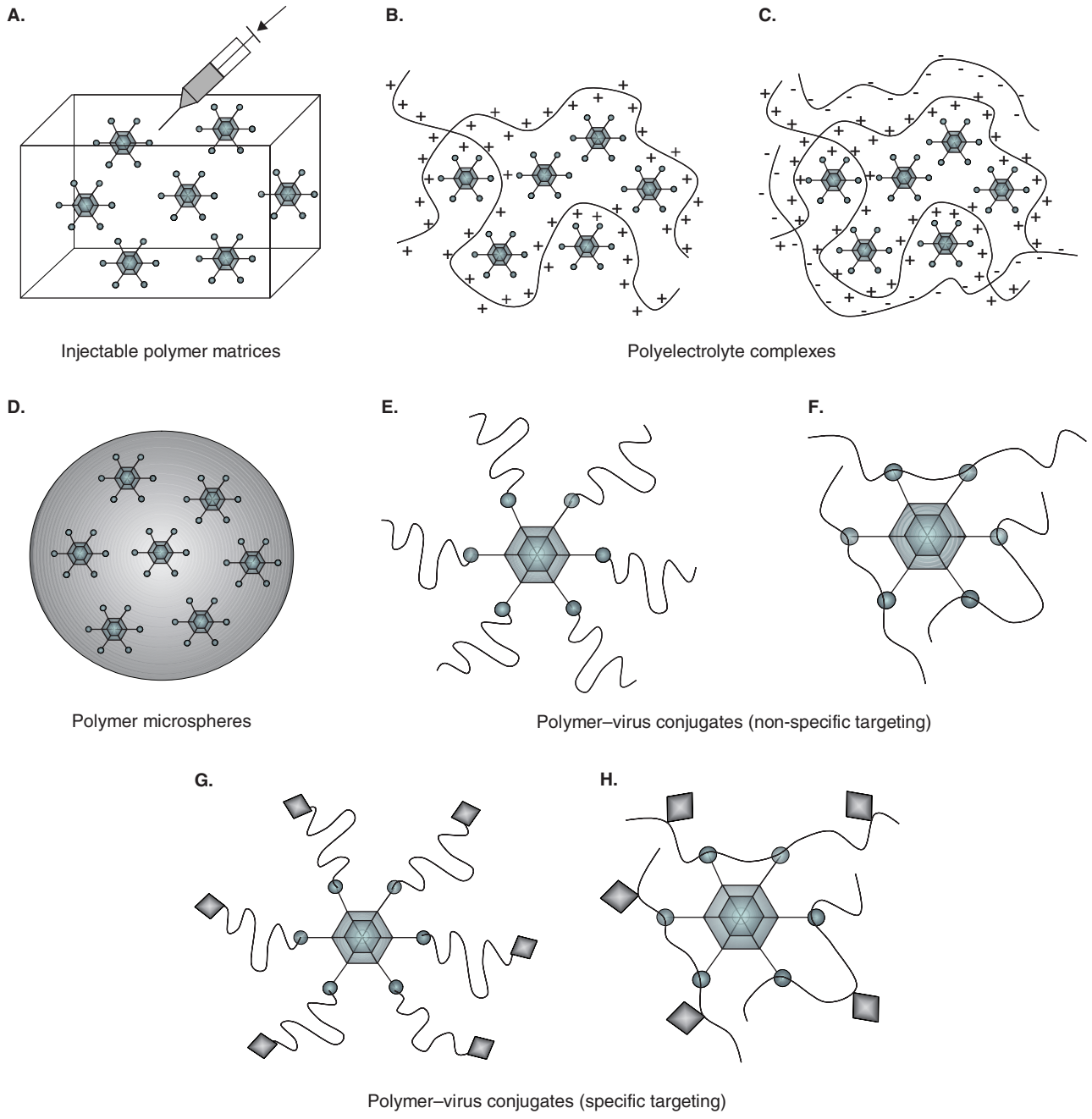
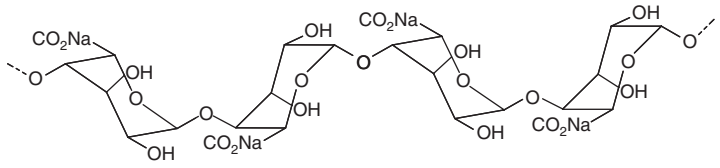
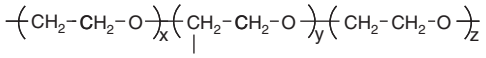


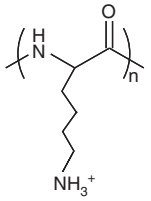
Figure 1. Schematic illustration of polymer-based approaches for viral gene delivery. Water soluble, biocompatible natural polymers such as collagen [40-45], alginate [46], Pluronics [47-51] and recombinant silk and elastin-like proteins (SELPs) [52] are used to entrap viral vectors that can be delivered through injection (**A**). Polycations such as polylysine, polybrene and polyethylenimine [35,54,59-64] and block copolymers [65] form polyelectrolyte complexes with negatively charged viral vectors (**B**), sometimes in combination with additional polyanions (**C**). Hydrophobic, biodegradable polyesters such as poly(lactic-*co*-glycolic acid) [71-75,77,78], or hydrophilic alginate mixed with calcium ion [79,80] form microspheres to entrap viruses (**D**). Linear water soluble polymers such as polyethylene glycol [86-91,94-96,98-101] and poly(*N*-(2-hydroxypropyl)methacrylamide) [110] can be conjugated chemically to the viral particle surface either at one end of polymer chains (**E**) or through multiple points of attachment via the polymer side-chains (**F**). These polymers can be further functionalized with cell-specific ligands to achieve targeting to specific cells and tissues (**G**, **H**) [92,93,106,107,111-115].



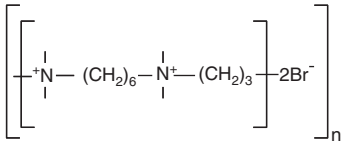
Alginate (Na salt)



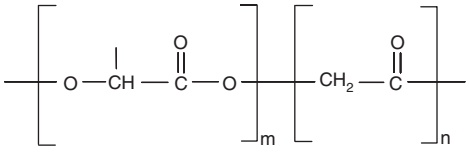
Poloxamer (Pluronic)



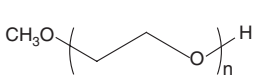
Poly(L-lysine)



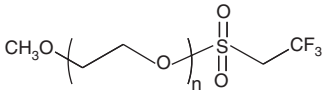
Polybrene



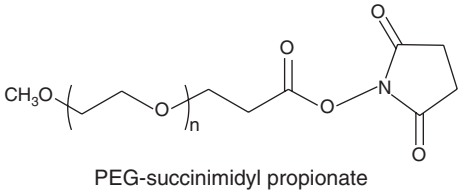
Poly(lactic-*co*-glycolic acid)



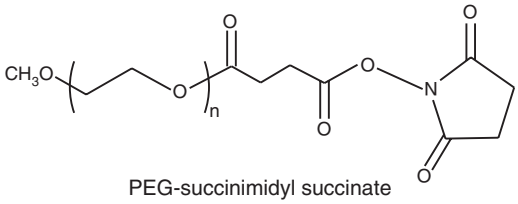
Methoxy PEG



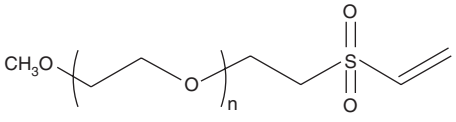
PEG-tresylate



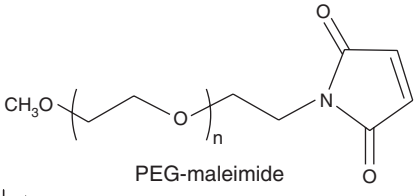
PEG-succinimidyl propionate



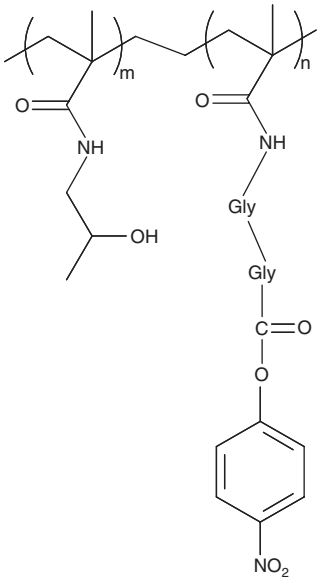
PEG-succinimidyl succinate



PEG-vinyl sulfone



PEG-maleimide



Poly[*N*-(2-hydroxypropyl)methacrylamide-*co*-diglycyl *p*-nitro phenyl ester]

Figure 2. Chemical structures of selected polymers used for viral gene delivery.

to inject virus/collagen into tissues followed by hydrogel formation *in situ*. For example, Ad vectors encoding growth factors such as platelet-derived growth factor-B were delivered from collagen hydrogels and enhanced wound healing *in vivo* [40]. Similarly, viral vectors delivered from collagen gels induced expression of tissue tropic factors in cells, which may have contributed to the repair of the anterior cruciate ligament [41]. Different types of polymers, or combinations of polymers, are also necessary for promoting healing of different tissues. For example, viruses released from fibrin gels, microporous gels, or composite gels of collagen and hydroxyapatite, are found to be more effective than collagen alone in promoting the healing of bone tissue [42,43]. Sometimes instead of using collagen, gelatin is used due to its higher solubility in water [44]. Collagen, and in particular, gelatin, may bind to viral vectors electrostatically, thus promoting the entrapment and retention of viral vectors in such hydrogels. Such matrix/virus interaction, however, is nonspecific. To increase the affinity and specificity of viral vectors toward the matrix, antibodies that bind to viral vectors were conjugated to the collagen matrix. As a result, the efficiency of viral vector loading into collagen can be improved and the release of viral vectors can be controlled [45].

Another popular type of injectable hydrogel for viral vector delivery is based on alginate, a natural polysaccharide. Alginate is highly water soluble and forms hydrogels upon exposure to divalent cations such as Ca^{2+}, which cross-links the alginate molecular chains through ionic bonding with the carboxylic groups. Alginate hydrogels containing entrapped viral vectors can be produced by injecting a mix of soluble alginate and virus into a bath containing cations, or by quickly mixing alginate, virus and Ca^{2+}, followed by immediate *in vivo* injection. Viral vectors delivered directly into tumor tissue by mixing with viscous alginate solution were found to have greatly reduced dissemination to other healthy tissues and organs. It is apparent that this was due to the highly viscous alginate gel reducing fluid convection in the interstitial tissue space [46].

Polymers that undergo reversible gel–sol transitions in response to temperature change between 4ºC and physiologic 37ºC have been investigated as injectable delivery systems for viral vectors. Poloxamers, or 'pluronics', are such temperature-sensitive polymers. Poloxamers are macromolecular surfactants composed of triblock copolymers of polyethylene oxide and polypropylene oxide of various length. Aiming for more effective cancer treatment, intratumoral infusion of viral vectors suspended in poloxamer hydrogel was found to reduce virus dissemination and significantly increase transgene expression in solid tumors [47]. The observed reduced spread of viral vectors to normal tissues was attributed to the increased viscosity of poloxamer 407 at body temperature, which had likely prevented circulation of viral vectors in the interstitial space and the lumen of microvessels around the infusion site. Poloxamers are also used for improving localized therapeutic viral gene delivery to vascular smooth muscle cells. The ability of poloxamers to maintain prolonged, high vector concentration around target cells was assessed *in vitro*. A significant increase in the number of cells transduced following brief exposure to Ad vector in the presence of poloxamer 407 was observed [48]. *In vivo* studies revealed similar benefits of using poloxamer 407 in delivering genes to balloon-injured rat carotid arteries. When the vector was delivered in poloxamer, the transfection efficiency of adenovirus-mediated arterial gene delivery was generally found to be significantly enhanced, while incubation time was reduced. Additionally, the use of poloxamer did not alter site specificity of gene transfer or evoke cell toxicity [49]. The usefulness of poloxamer 407 was further tested for percutaneous Ad-mediated gene transfer in vascular stents. As expected, in the presence of poloxamer, a significant increase in the level of gene expression can be achieved with reduction in transduction time when gene transfer was performed pre- or post-stent implantation [50]. Other types of poloxamers such as F127 had demonstrated use as carriers for HIV-1 lentiviral vectors expressing green fluorescent protein to the central nervous system. Although similar transduction efficiency of astrocytes was observed with or without gel *in vitro*, stereotaxic delivery of viral vector in 15% F127 to the rat brain resulted in localized transduction of cells [51].

More recently, a new type of biomimetic, genetically engineered protein hydrogels, has been used for delivering adenoviral vectors to solid tumors [52]. Recombinant SELP polymers were designed to incorporate typical sequences from silk (alanine-glycin repeats) and elastin (glycine-valine-glycine-valine-proline repeats). Some of the valine residues were also replaced with lysine, whose positively charged side-chain may interact with viral particles. Ad vectors delivered in SELP hydrogels generated gene expression *in vitro* that lasted for four weeks. After intratumoral injection, localized gene expression persisted for over two weeks without much dissemination outside the tumor area. These results indicate high potential for SELPs, whose temperature-sensitivity and biocompatibility are well-documented, for use in viral gene therapy for cancer.

An alternative to the use of physiological temperature as the trigger for hydrogel formation is an external light source [53]. In one example, gelatin was modified to contain photo-reactive vinyl groups, then mixed with Ad vectors and injected into the body after the surgical removal of malignant tumors. Gelatin hydrogels were formed *in situ* through polymerization initiated by external UV light. Local and prolonged release of anti-tumor Ad vector showed promise for preventing the recurrence of cancer after surgery.

5. Polyelectrolyte complexes

The coating of viruses with cationic or, to a lesser extent, with a combination of cationic and ionic polymers has been

found to significantly improve vector performance by enhancing infectivity and gene expression even in cell lines that are normally resistant to adenovirus infection [54-57]. It has been hypothesized that polycations enhance gene transfer *in vitro* by reducing the electrostatic repulsion between both negatively charged viral particles and cell surface, thereby improving binding and uptake of vectors. While the idea of complexing adenoviruses with polycations is mainly to improve transduction in cells resistant to Ad infection [31,55,56], complexing retroviruses with polycations stems from the need to increase the rate of the virus reaching target cells in general [35]. The inability of retrovirus to transfer sufficient gene for effective therapeutic application has been attributed to the slow rate of virus binding to cells; by the time the viruses reach the cell surfaces, more than 90% have lost their bioactivity [34].

Kaplan and co-workers tested the efficiency of Ad-mediated gene transfer in the lungs using vectors complexed with different polycations including polyethylenimine (PEI), hexadimethrine (polybrene, PB), protamine, poly-L-lysine (PLL) and DEAE-dextran. Each type of viral polyplex gave rise to increased levels of transgene expression in the lungs compared with the same dose of vector administered alone [54]. In all cases, increased transgene expression was observed in cells previously determined to be resistant to Ad infection, such as those of the trachea and upper airway [54,58]. Use of cationic lipids lipofectamine and DOSPER (1,3-dioleoyloxy-2-(6-carboxyspermyl)propyl-amide) [55] in Ad-mediated gene transfer showed a similar transduction enhancement effect in addition to providing the vectors with partial protection against neutralizing antibody *in vitro* [57].

A series of studies [35,59-63] have focused on complexing retroviruses with charged polymers as a means to increase the rate of viral delivery to the target cell surface and enhance gene transfer. Coating of retroviral surfaces with cationic polymers generally enhanced infection, according to the authors, by possibly reducing the electrostatic repulsion between both negatively charged retroviruses and the cell surface. In fact, some anionic polymers were found to reduce transduction efficiency [63,64] while others, including chondroitin sulfate proteoglycans and glycosaminoglycans (GAGs), inhibited retrovirus transduction [59,60]. Interestingly, the combination of a low dose of anionic GAGs with equal weight concentration of cationic GAGs enhanced rather than inhibited transduction efficiency, with polyplexes containing 80% of viable viruses [61].

Another contributing mechanism of enhanced viral gene transfer by polyelectrolyte complexation is virus aggregation. Davis *et al.* have systematically studied this effect on retroviral gene transfer by using polylysine of different molecular sizes [62]. They found that while all sizes of polylysine enhanced gene transfer by charge shielding, only polymers of at least 15 kDa contributed to virus aggregation, forming large particles averaging 1 to a few µm in diameter. Larger, heavier, charge-shielded viral polymer aggregates were able to reach the cell surface faster than small particles and were taken up by cells effectively. Formulation of retroviral complexes coated with poly(ethylene glycol)-poly-(L-lysine) block copolymer (PEG-PLL) were similarly found to improve viral infectivity and transduction efficiency in Lewis carcinoma cell lines and in primary cultured brain cells without increasing toxicity [65]. PEG's high affinity to biomembranes combined with cationic PLL's ability to neutralize negative charges, help yield vectors with stably modified surface and enhanced gene delivery performance [65]. Although no characterization of physico-chemical properties of the complexed virus was reported, this interesting work could potentially lead to a new, simple approach of coating single viral particles and retargeting them.

Despite all of the reported benefits, polycation-based gene delivery systems have limited utility in the systemic delivery of therapeutic genes due to difficulties in formation, *in vivo* stabilization, ease of aggregation and precipitation, toxicity and low transfection efficiency [66,67]. An effort to overcome these limitations focused on development of biodegradable polymers such as gelatin with additional positive charges [68,69]. Thus, conjugation of gelatin with cationic molecules such as ethylenediamine allows for control of positive charge ratio per gelatin molecule. Such biocompatible cationized gelatin complexed with HVJ (hemagglutinating virus of Japan) envelope vector showed enhanced gene transfection efficiency both *in vitro* and *in vivo* [68].

6. Polymer microspheres

The polyester family of copolymers of lactic and glycolic acid (PLGA) is the most commonly used biodegradable polymer for fabrication of microspheres for drug delivery [70]. PLGA is well-characterized and widely investigated for implantable devices due to its excellent biocompatibility. Upon hydrolytic degradation, PLGA is converted to lactic and glycolic acids, both natural metabolites of the human body, with minimal inflammatory responses.

There has been a long-lasting interest in encapsulating Ad vectors in PLGA microspheres. Due to the small size of microspheres, administration via injection is straightforward. On the other hand, microspheres are large enough to persist locally without disseminating to remote tissues and organs. It is possible to achieve prolonged release of viruses through controlling microsphere degradation. Microspheres are also expected to enhance viral transduction in certain cells, in particular, phagocytic cells. In an attempt to improve the effectiveness of Ad-mediated gene transfer for glioma therapy, PLGA microspheres encapsulating Ad vector, ranging in size from 100 to 200 µm, were prepared using a double-emulsion technique [71]. Microspheres were found to mediate sustained release of low dose adenovirus up to over 10 days and achieved a level of gene transfer that could reduce tumor burden. PLGA microencapsulation also

reduced antigenicity of Ad following *in vivo* delivery. However, the process of encapsulation was found to be inefficient, yielding only 10% of input virus in microspheres and releasing only 10% of encapsulated virus [71]. In a recent study, Ad vector encapsulated in PLGA microspheres achieved approximately 25% encapsulation efficiency, of which more than 10% of the virus retained functional activity [72]. *In vitro* release experiments showed a slow release of 15% in 11 days. Despite the low loading efficiency and release of virus, the PLGA microspheres delivered a model DNA vaccine to antigen-presenting cells and elicited robust antigen-specific immune responses *in vivo*, while minimizing virus dissemination and undesirable immune responses toward the Ad vector itself. In another study, high encapsulation efficiency (23%) of Ad vector in PLGA microsphere was achieved, but it was acknowledged that the processing conditions and changes in environmental factors may have influenced the transfection ability of encapsulated Ad [73]. Interestingly, virus encapsulation efficiency appeared to relate to the size of microspheres, with higher encapsulation in smaller microspheres [74]. On the other hand, large microspheres help to reduce immunogenicity of the virus [75]. Attempting to improve the yield of viable virus in PLGA microspheres, Matthews and co-workers co-encapsulated Ad in poly-L-lysine and PLGA. Cationic PLL addition increased the efficiency of gene transfer following the release of virus from the microspheres, but did not improve virus yield in formulation or virus release [76]. Del Barrio *et al.* reported a mild method of virus encapsulation using PLGA [77]. The conventional emulsification method subjected the viruses to vortex which resulted in large particles and, as previously reported, low virus yield. In a novel method called total recirculation one-machine system (TROMS) [78], the emulsification step relies on the turbulent injection of the phases, rather than vortex. Although fewer viruses were encapsulated in TROMS, the level of infectivity was interestingly higher. This was attributed to a number of possibilities, including the mild method of encapsulation, which could better preserve the activity of viruses, and perhaps the development of immune tolerance due to slow release of vectors. Encapsulated Ad was also found to be protected from inactivation by serum factors [79].

Microspheres made from alginate physically cross-linked with Ca^{2+} ions and reinforced by polylysine are also useful carriers for viruses. One earlier report showed that reovirus encapsulated in alginate microspheres could be delivered orally to neonates and helped the virus to avoid neutralizing maternal antibody [79]. In comparison to the double-emulsion method of preparing PLGA microspheres, microencapsulation in water soluble alginate does not expose the virus to organic solvent which could potentially damage viral structure. In a typical procedure, viral particles are mixed with sodium alginate in water with surfactant and canola oil, emulsified and Ca^{2+} ions are added. Microspheres are then spun down, washed and further coated with a layer of polylysine. One example of Ad vector encapsulated as such was tested as a vaccine after systemic and mucosal delivery [80]. Successful delivery of the virus was demonstrated in all routes of administration, with significant production of antigen specific IgG and IgA. In particular, intranasal using alginate microspheres only used 40% of the dose compared to other routes, suggesting alginate microspheres may be particularly useful for intranasal delivery [80].

Based on the simple binary alginate–polycation system, a much more complicated sub-micron particle formulation for Ad vector was developed [81]. Ad vectors were embedded within polymer particles of roughly 230 nm in size, using a number of reagents including sodium alginate, cellulose sulfate, spermine, poly(methylene-*co*-guanidine), calcium chloride and F-68 (a poloxamer as surfactant). The formulation yielded very stable colloidal particles, which maintained infectivity over a year of storage at -80°C, and were also mechanically robust. These particles are presumed to degrade in the presence of extracellular or intracellular hydrolases, and they transfected cells efficiently over an extended period of time [81]. Other microsphere approaches similar to the alginate/Ca^{2+} system have been developed that exploit the complexation or coacervation of oppositely charged molecular species such as gelatin/alginate [82] and chitosan/bile salt [83].

While most microspheres encapsulate viruses, one research group reported a system in which biotinylated Ad vectors were attached only to the surface of streptavidin-coated 0.5-µm silica microbeads [84]. Microbeads restricted the movement of Ad vectors, and thus localized and further enhanced gene transduction as compared with unmodified Ad vectors. Furthermore, Ad-microbeads coated with biotinylated Concanavalin A enhanced specific targeting to cells. It was postulated that this delivery system could be generally applied to other vectors and other cell targeting ligands.

7. Polymer–virus conjugates

Direct, chemical modification of viruses with water soluble polymers aims to enhance transduction efficiency, improve cellular specificity and reduce immunogenicity and toxicity of the viral vectors. This strategy is inspired by the well-established paradigm that polymer-conjugated small molecular drugs and protein drugs show higher stability against degradation and nonspecific clearance *in vivo*, which translates into higher efficacy [85]. Over the years, various methods and polymers employed for protein modification have been adapted to modifying viruses. Here we review two major strategies of using mono-functional polyethylene glycol (PEG) and multi-functional *N*-(2-hydroxypropyl) methacrylamide copolymers (polyHPMA).

7.1 PEGylation

Modification of viruses using PEG confers vectors with the ability to evade both innate and acquired immune response.

Targeting viral vectors are also being developed using PEG to introduce a wide range of ligands. One attractive attribute of PEG is its low toxicity and non-immunogenic nature, which is evidenced by its widespread use in foods, cosmetics and pharmaceuticals approved by the US Food and Drug Administration (FDA). Methods of PEGylation have been well-established in recent literature. A wide range of commercially available mono-functional PEG derivatives have been used for PEGylation of viruses. These include amine-reactive molecules such as PEG succinimidyl succinate, PEG tresylate, PEG succinimidyl propionate and thiol-reactive molecules such as PEG vinylsulfone and PEG maleimide. Mono-functional PEG allows for the facile conjugation of polymer to the viral capsid, while homo- or hetero-bi-functional ends permit simultaneous introduction of PEG and targeting ligand.

7.2 Mono-functional PEG

The chemistry of PEGylation has been optimized over the years to endow Ad vectors with the ability to escape neutralizing antibodies and retain infectivity both *in vitro* and *in vivo*. Conjugations of monomethoxy-PEG (mPEG), activated with either cyanuric chloride (CC-mPEG), succinimidyl succinate mPEG (SS-mPEG), or tresyl-MPEG (TmPEG), to lysine residues of viruses have shown that TmPEG was superior over CC-mPEG and SS-mPEG in retaining high titers following production and infectivity [86,87]. These observations have been attributed to the mild conditions required for the coupling reaction using TmPEG allowing for greater retention of bioactivity. Succinimidyl propionate mPEG (SPA-mPEG) can also be used to modify vectors without any adverse effect on transduction efficiency. PEGylation provides effective shielding for viruses against neutralizing antibodies and is utilized by many to develop viral vectors with the ability to evade immune responses [88-91]. Modified first generation Ad vector (FG-Ad) showed enhanced physical stability of the vector allowing for less stringent storage conditions and ease of handling, thus giving this technology an added advantage [88].

The degree of PEGylation however, does have profound influence on the biological performance of viral vectors. Higher levels of PEG-Ad modification are generally expected to better protect and shield the viruses [92], although the attached polymers may hinder the vectors' ability to enter cells [93,94]. Mok *et al.* noted that while heavily PEGylated FG-Ad and helper-dependent Ad (HD-Ad) may ablate transduction *in vitro* due to over-modification, which prevents vectors from binding to CAR, they still retain the ability to infect cells *in vivo* [95]. According to Eto *et al.*, most studies eliminated PEG-Ad from *in vivo* tests once they were determined to have poor infectivity *in vitro* [92]. Levels of modification in the range of 34 – 70% were shown to be sufficient in protecting the virus from neutralization in the immune serum while still allowing for efficient transduction. Along this line, it is recently reported that PEG-Ad with about 90% modification showed much higher gene expression in an *in vivo* tumor model and greatly reduced expression in the liver [96]. In this case, it was postulated that Ad with a very high degree of PEGylation achieved long systemic circulation and localized in the tumor due to the enhanced permeability and retention (EPR) effect [97].

Apart from their ability to evade neutralizing antibodies, PEGylated Ad is conferred with the ability to reduce innate and cellular immune responses as well, without much compromise on cells transfection *in vivo*. Numerous studies have demonstrated the ability of PEG-Ad to reduce cytotoxic T lymphocyte response, allowing for partial re-administration of native virus or viruses modified using different activated mPEG [88,98]. Re-administration of PEGylated HD-Ad vector to mice immunized with unmodified Ad also demonstrated the ability to produce a significant level of transgene expression in the liver [98,99]. PEGylated FG-Ad and HD-Ad were further assessed as having the capacity to reduce innate immune responses through decreasing levels of a pro-inflammatory cytokine IL-6 generated upon administration of the vectors *in vivo* [92]. Mok *et al.*, suggested that PEG-Ad reduced nonspecific uptake of vectors into macrophage and Kupffer cells, thereby lowering cytokine IL-6 levels, without affecting vector uptake in other cells or tissues [95]. In a separate study, it has been shown that PEG-Ad mediates levels of transduction similar to those of unmodified Ad in liver, lungs, spleen, peritoneal membrane and kidneys [92]. The indiscriminative ability of PEG-Ad to infect different cell types unfortunately accounts for the observed tissue damage. Several studies have shown that PEGylation of FG-Ad had no effect in reducing liver damage [92,99]. PEGylation of HD-Ad, on the other hand, provided both liver protection and an improved safety profile [100]. Recently, it was found that PEGylation also improves blood compatibility of Ad vectors by reducing the activation of platelets and endothelial cells [101].

Similar attempts to modulate physical and immunogenic responses of other viral vectors including adeno-associated virus [89,90] and baculovirus [91] have also relied on PEG and methods of PEGylation. Common characteristics of PEGylation were observed for all modified viruses: PEGylation of adeno-associated viral vectors using SPA-mPEG gave rise to a moderate level of protection of vector against antibody neutralization [89]; PEG-decorated cowpea mosaic virus was effectively shielded from inducing a primary antibody response [102]; PEG-derivatized baculovirus had increased transduction efficiency both in the lungs and brain [91].

To summarize, PEGylation tested in different viral systems is proven to be effective in rendering the vector less susceptible to inactivation by neutralizing antibodies, allowing for extended plasma circulation time *in vivo*. The demonstrated efficiency of PEG in reducing host immune responses toward virus particles permits repeated

administration of PEG HD-Ad. Surface PEGylation has seen reduced interaction of vectors with their natural receptors, which is of added advantage for the design of vectors with efficient de-targeting and retargeting ability.

7.3 Bi-functional PEG

Given all the benefits of PEGylation, viral vectors often lack the crucial ability to efficiently express gene in diseased tissues without affecting the healthy tissues. Clinical realization of gene therapy will also depend to a large extent on the development of vectors with a high degree of cellular specificity. Toward this end, bi-functional PEG was developed. It allows for attachment of viral vector to one end and a cell-specific targeting ligand to the other. Strategic use of bi-functional PEG in combination with the understanding of the mode of interactions between viruses and cell receptors may significantly aid in the design of vectors with improved targeting ability.

For instance, it has been established that infection of Ad is generally initiated through interaction of the viral knob with CAR, followed by cell attachment [103,104]. Internalization is then facilitated by interaction of the RGD motif at the penton base of the virus with cell integrins [105]. PEGylation of Ad, however, ablates CAR binding and, in effect, reduces the vector's ability to enter cells. Lower transduction efficiency of Ads in response to increasing levels of PEGylation (at least *in vitro*) has been documented [91]. However, limited cell entry of PEG-Ad via CAR binding could be regarded as a benefit in the design of vectors with improved cellular specificity since CAR receptors are ubiquitously expressed in many different cell types that permit delivery of therapeutic genes to unintended cells. Incorporation of a 'targeting ligand' to PEG-Ad should in principle, produce a complex that recognizes only receptors over-expressed on the surface of diseased cells and has reduced tropism for the native Ad receptor CAR [94]. Lanciotti *et al.* have demonstrated the ability of an Ad vector modified with fibroblast growth factor (FGF2) to bind more selectively to cells expressing FGF2 receptor and exhibit enhanced efficiency of transduction both *in vitro* and *in vivo*. Non-target tissues such as the spleen and liver display a marked decrease in transduction following *in vivo* delivery of the modified vector, which still retain the ability to evade neutralizing antibody and display reduced T cell activation [94]. Given that cell entry requires interaction of RGD and integrins, Eto *et al.* constructed PEG-Ad vectors with RGD peptides on the tip of the PEG and was able to remedy the low infectivity due to PEGylation in various cell types of the lung, spleen, kidney, heart and brain [93]. They thus demonstrated the use of functional groups on the tip of PEG to effectively change adenovirus tropism. Xiong *et al.* [106] extended the use of RGD-PEG to specifically target $\alpha_v\beta_3$ integrins that are highly expressed in tumor cells and demonstrated that conjugation of RGD-PEG to FG-Ad led to enhanced tumor cell infectivity with a minimum level of transduction in non-target liver tissues.

Introduction of targeting ligand via heterofunctional PEG is further adapted for use in combination with genetic modification of viral capsid to allow for introduction of short cysteine-containing motif. The introduced cysteine groups allow for simultaneous de-targeting by PEGylation and retargeting by coupling of thiol to activated PEG and to targeting ligand, such as maleimide-PEG-transferrin [107]. The use of transferrin (Tf) was intended to increase the vector's affinity to tumor cells expressing transferrin receptors (TfR). Thus, Tf was first reacted with maleimide-PEG-*N*-hydroxysuccinimide, resulting in maleimide-PEG-Tf, which was then coupled to cysteine mutants of Ad vector (Ad1Cys) to form Tf-PEG-Ad1 [107]. The tumor-targeted Tf-PEG-Ad1 could be further subjected to more PEGylation. The resulting vector was physiologically stable and could still provide effective tumor-specific targeting.

To summarize, bi-functional PEG provides a convenient and simple approach to retarget viral vectors without the need for substantial genetic modification of the vector. A range of ligands can be linked to the virus particles through reactive groups such as maleimide or active esters at the ends of the PEG molecule. This approach allows for development of vectors with improved targeting ability, enhanced pharmacokinetic profiles associated with PEGylation and minimized non-specific uptake of vector by non-target cells.

7.4 Multi-functional polyHPMA

Unlike PEGylation of viruses, where polymer chains are attached to viral vectors through single points, multi-functional polymers with numerous chemically reactive side-chains represent another approach of generating polymer–virus conjugates. Research in the past has focused almost exclusively on the use of a water soluble synthetic copolymer of *N*-(2-hydroxypropyl)-methacrylamide (polyHPMA) bearing amine-reactive side-chains of *para*-nitro phenyl ester (ONp). The HPMA copolymers are widely investigated as a water soluble macromolecular carrier for anti-cancer drugs and are being tested in clinical trials [108]. The synthesis, characterization and biocompatibility of this class of polymer have been extensively investigated and well-documented over the past three decades [109]. The idea of using multi-functional polyHPMA for viral modification was put forth in 2001 by Seymour and his group [110]. In this study, a random copolymer of HPMA and 10 mol% ONp with an average molecular weight of 16,500 was chemically conjugated to Ad particles. Multi-functional polymer coating abrogated the infectivity of the virus, which clearly differed from the mono-functional PEGylation approach. It was also nicely demonstrated that further conjugation of cell targeting ligands of FGF and vascular endothelial growth factor (VEGF) enabled retargeting of polymer coated Ad vector to cells expressing receptors for

FGF and VEGF. Modification with polyHPMA also enabled Ad vector to evade neutralizing antibodies *in vitro* and *in vivo*. A series of publications that followed the 2001 report further confirmed that polyHPMA modification is capable of improving viral vector stability, enhancing cell specific targeting, avoiding nonspecific clearance by the immune system and achieving significant gene expression both *in vitro* and *in vivo* [111-115]. Importantly, impressive results were reported for selective targeting to tumor tissue while minimizing nonspecific uptake and gene expression in the liver, using either non-targeted polymer coating to exploit the EPR effect or retargeted polymer coating using peptide and protein ligands [114,115].

8. Conclusions

To address the weaknesses of viral gene delivery, a variety of polymer-based systems have been developed to help overcome undesirable immune responses, and to improve stability, gene transduction efficiency and *in vivo* cell-specific delivery of viral vectors. Polymers can be engineered into injectable matrices or microspheres for entrapping viruses, forming polyelectrolyte complexes with viral particles, or direct chemical conjugation with viral particle surfaces. The functions of the polymers are to physically shield viruses from immune surveillance and degradation, provide a virus depot for prolonged gene transfer, minimize nonspecific sequestering and clearance from the body, and retarget viral vectors to specific cell targets. Significant progress has been made in establishing feasibility of these approaches *in vitro* and demonstrating efficacy in preclinical animal models of cancer and other diseases.

9. Expert opinion

Viral vectors remain by far the most efficient means of mediating gene transfer. The sophistication and effectiveness of the viral structure and function in overcoming gene delivery barriers at the systemic and cellular scales are unmatched by even the most advanced nonviral gene delivery systems known to date. Since the early years of viral gene therapy, tremendous progress has been made in genetic engineering and modification of viral vectors, aiming to overcome major concerns related to the risk of viral replication and infection, insertional mutagenesis of the host genome, adverse immune responses, structural instability, difficulty and the high cost of manufacturing, transgene packing and storage, and limited efficacy in targeting specific cells and tissues without harming healthy tissues. While novel and improved forms of viral gene therapy are being developed at a rapid pace, such as the advent of highly effective oncolytic viruses for the selective destruction of cancer cells [116], many problems related to viral gene delivery remain unsolved. On the other hand, nonviral gene carriers, in particular polymer-based systems, are also progressing rapidly, but have not yet reached a point to have any significant impact on clinical translation of gene therapy as viral vectors do [38]. Nonetheless, knowledge and experience gained from polymer-based delivery of small and large-molecule drugs can be applied toward the design and delivery of viral vectors. Furthermore, even polymer systems developed for cell delivery and encapsulation can be adapted to viral delivery. However, it is important to note that although polymer-based approaches may significantly enhance viral gene delivery, such as mitigating immune responses against viruses, flaws due to the viral vectors themselves, such as uncontrolled integration into host genome, are probably not amenable to improvement by polymers. Moreover, even though polymers may be effective in protecting viral vectors against neutralizing antibodies, viral proteins may still elicit cellular immune responses after uptake and degradation by target cells.

One important aspect of improving polymer-based viral gene delivery is to optimize the manufacturing process and to maximize the bioactivity of viral vectors by inflicting minimal damage on the viral structure. This is particularly relevant to approaches using polymer microspheres, since both injectable polymers and polyelectrolyte complexation are already done under very mild conditions. The typical double-emulsion process of virus encapsulation applied in forming polymer microspheres often exposes viruses to organic solvent and high shear stress that may cause damage to their structure. The loading efficiency of such procedure is often low. Careful optimization of the processing conditions and, perhaps, innovative ways of generating microspheres without the use of organic solvent and high shear force may eventually lead to renewed interest in virus microencapsulation. Compared to the classic water insoluble polyesters, hydrophilic biopolymers such as alginate, which has been extensively used for microencapsulation of cells, may be more appealing materials for viral encapsulation. Finally, the latest developments in microfluidics [117] and nanofabrication may encourage innovative ideas of packaging viral particles in micro- and nano-scale polymer capsules.

While much can be accomplished by modifying existing polymer systems, new polymers and chemistries are needed to further improve the efficacy of viral vectors. For example, different routes of administration of viral particles present unique challenges and demand different polymer delivery systems. Oral delivery of viral vectors is a highly desirable method, but most viruses do not survive the harsh environment of the gastrointestinal tract, including the acidic pH in the stomach and intestines filled with digestive enzymes. Because of the difficulty in protecting viruses against the GI tract, only a few published reports exist that describe the use of PEGylation and enteric polymers for oral delivery of virus [118,119]. Much more needs to be done to create responsive polymer constructs that are protective during

transport along the gastrointestinal tract but deliver fully active virus across particular segments of the gut.

The dual challenge of constructing a polymer delivery system that can evade clearance and be able to release its virus cargo upon reaching target cells may be tackled with molecular engineering of biodegradable and bio-responsive polymers. As a simple example, the chemical linkage of a PEG chain to a viral particle may be manipulated to optimize the biological activity of the virus *in vivo*. If the linkage is a stable amide bond, the PEG chains are more likely to persist for longer periods of time *in vivo*, rendering the viral particle less effective in infecting cells. If the PEG is conjugated through an ester bond, then it is expected to degrade in the body, perhaps even more quickly in the cell endosome, thereby enabling the viral vector to recover its infectivity. In the future, it is conceivable to design other chemical linkages that remain stable in normal physiologic milieu, but labile in response to biological signals such as pH fluctuation, re-dox environment, and/or enzymatic activities when the virus needs to be released extracellularly or intracellularly.

Detailed characterization of polymer–virus constructs and elucidation of the structure–property relationship of polymers are essential for better understanding the existing delivery systems as well as developing new systems. In order for injectable polymer hydrogels to effectively prevent viral dissemination to healthy organs, and still allow for controlled release of virus to infect target cells, what are the optimal parameters for hydrogel such as the mesh size, mechanical strength, stability and rate of degradation? How can specificity be introduced into the hydrogel material to precisely control the incorporation and release of virus? While polyelectrolyte complexation is very effective in enhancing viral transfection *in vitro*, it is difficult to be used directly *in vivo* due to the inherent instability of these polyelectrolyte colloidal particles. Can polyelectrolyte complexation be conducted in ways to generate sterically stable polymer/virus constructs with defined structure and size? Conventional PEGylation chemistry is nonspecifically reactive to amino acids on viral particles, which may lose bioactivity if viral proteins essential for infection are chemically modified. Can PEGylation be carried out in site-specific manners using more elaborate chemistry such as the 'click' reaction [120]? Can polymer conjugation be made reversible depending on external stimuli (such as photo-irradiation [121] or internal stimuli (such as proteolysis [122])? Can we develop new characterization tools to analyze the molecular details of the polymer/virus constructs, such as how many polymer chains are attached to an average viral particle and at what locations of the viral capsid [123]? Finally, as new viral gene therapy products are being translated to clinical applications [124], development of effective, biocompatible, regulatory friendly, inexpensive polymer systems to aid in viral gene delivery will have a bright future, with many opportunities leading to technological innovation, scientific discovery and ultimately more clinical translations.

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