Review

Designer adenoviruses for nanomedicine and nanodiagnostics

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With the recent explosion of interest in the field of nanobiotechnology, viruses are now finding new applications in materials science and medicine. Here, we discuss the engineering of 'smart' nanoparticles that are based upon recombinant adenovirus (Ad) vectors and that combine multiple functions of targeting, imaging and drug delivery. We focus on the use of Ads as a carrier and delivery system for macromolecules other than DNA and develop the rationale behind using Ads for such applications. Due to the modular nature of the Ad capsid, multiple therapeutic or diagnostic modalities, such as the addition of magnetic resonance imaging contrast agents, radiation sensitizers and antigenic peptides for vaccines, can be incorporated by modifying different sites on the viral capsid. These types of particles have a tremendous potential to increase the sensitivity and specificity of therapies.

Introduction

The study of viruses traditionally has focused on their roles in infection and disease, and over the past two decades, also their use in gene therapy. Viruses are now finding new applications in materials science and medicine [1]. They represent near ideal nanoparticles due to their regular geometries, well characterized surface properties, nanoscale dimensions and, uniquely, their structure being known to near atomic resolution [2]. They can serve as biocompatible scaffolds to which a wide variety of inorganic and biological structures can be attached [3]. Their large size relative to biomolecules such as polypeptides and antibodies and their high ratio between surface area and volume allows for attachment of multiple moieties to specific sites. Molecules can be incorporated onto the viral surface with control over their spacing and orientation [4], and this can be used to add reactivity to specific sites of the capsid.

Recombinant adenoviruses (Ads) have shown immense promise for gene therapy because they are extremely efficient at delivering DNA to target cells, can infect both dividing and quiescent cells, have a large capacity for incorporation of cDNA expression cassettes, and have a low potential for oncogenesis because they do not insert their genome into the host DNA [5]. Ads have received extensive clinical evaluation and are used for one-quarter of all gene therapy trials (http://www.wiley.co.uk/genetherapy/clinical). Disadvantages include a lack of potential for long-term transgene expression, the inherent hepatic tropism of intravascularly administered Ads, which precludes targeted delivery to alternative organs or disease sites, and induction of a strong cellular and humoral immune response and, potentially, a toxic inflammatory response. Several excellent articles have recently been published detailing interactions between Ads and their hosts, summarizing the blood interactions responsible for hepatic uptake and discussing adenoviral pharmacokinetics [6–8]. A brief description of Ad biology and structure is provided in Box 1.

In this review, we discuss the characteristics that make Ads particularly suitable for use in generating so-called 'smart' nanodevices for multimodal delivery of therapeutic and imaging agents, far beyond applications in gene therapy. We analyze how Ads, through nanoscale manip-

Glossary

Photodynamic therapy: PDT is a tumoricidal combination of visible light and a non-toxic, light-absorbing molecule called a photosensitizer. After absorption of light, the photosensitizer generates reactive oxygen species such as super oxide, which in turn react with proteins, nucleic acids and membrane lipids in the cell, leading to damage of proteins and DNA and disruption of the cell membrane. If the damage is too severe for the cell's repair mechanisms to overcome, the cell will then undergo apoptosis and die.

Photothermal therapy: PTT is closely related to PDT and involves the experimental use of electromagnetic radiation, usually in the form of visible light, for cancer therapy. However, PTT does not require oxygen to interact with the target cells or tissues and allows the use of light of a longer wavelength, which is less harmful to healthy cells and tissues. Instead, the photosensitizer is excited with a specific wavelength of light that causes it to enter an excited state in which it releases vibrational energy that, in turn, is transformed into heat. Because cancer cells are known to be particularly sensitive to heat, PTT can preferentially kill cancer cells while sparing normal tissue.

Radiation therapy: this therapy involves the use of an external ionizing radiation beam that causes damage to cellular proteins and nucleic acids, either directly through induction of DNA strand breaks or indirectly by ionization of water, which leads to the formation of free radicals such as reactive oxygen species, which subsequently cause damage leading to cell death.

Zeta (ζ)-potential: ζ-potential is a surface property of a particle suspended in a medium and describes the difference in electrical potential between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. The ζ-potential indicates the degree of repulsion between similarly charged particles or the attraction between oppositely charge particles in a dispersion. Generally, particle dispersions with a ζ-potential greater than +30 mV or less than −30 mV are considered stable due to the high degree of repulsion between particles. A ζ-potential between +30 mV and −30 mV indicates a tendency for the particles to aggregate, with the greatest degree of aggregation found at the dispersion's isoelectric point, a ζ-potential of 0 mV.

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Box 1. Adenovirus vectors

There are 51 distinct types (serotypes) of human adenovirus (Ad) based upon their ability to resist neutralization by antibodies elicited against other types of Ads. Each serotype is further placed into one of six groups, A–F, according to their ability to agglutinate red blood cells [80]. Ad infection is associated with the common cold, which leads to mild respiratory illness in normal adults, but depending upon the infecting serotype, it also can cause conjunctivitis, cystitis and gastroenteritis.

Ads consist of a non-enveloped icosahedral particle composed of 12 distinct proteins and an ~36-kilobase double-stranded linear DNA genome. A detailed description of Ads can be found elsewhere [81]. Briefly, Ads have a diameter of 60–90 nanometers (nm) and a total mass of 125 megadaltons (MDa). As seen in Figure 1, the principal capsid proteins are hexon (130 kilodaltons [kDa]), penton (82 kDa) and fiber (62 kDa), in addition to some hexon- and penton-stabilizing proteins, such as the 14 kDa protein IX (pIX). The 12 hexon trimers form each of the 20 facets of the virus, with 720 hexon monomers per virion. Central to each facet are groups-of-nine (GON) hexons that are believed to be stabilized by pIX, which is present in 240 monomer copies per virion. At each of the twelve vertices is a penton complex, which consists of a homopentameric penton base that is non-covalently attached to the trimeric fiber protein. A polypeptide loop containing an Arg-Gly-Asp (RGD) sequence extends from the penton base and is responsible for Ad binding to secondary cell receptors ($\alpha_v\beta_{3/5}$ integrins), triggering endocytosis of the virus. The fiber trimer extends 32 nm outward from the capsid. Each fiber monomer can be divided into three portions, a tail responsible for attachment to other capsid proteins, a long, flexible shaft and the knob domain, which is responsible for the high affinity binding to the coxsackie and adenovirus receptor (CAR).

ulation of the viral surface, can be used as templates for delivery systems that are suitable for carrying and targeting almost every type of therapeutic or imaging agent, such as inorganic, biological and radiological compounds, to disease sites. Furthermore, we examine how such modifications might be used to alter the colloidal and surface properties of Ads. Finally, we discuss some of the unique opportunities that the engineering of the Ad capsids could offer to produce novel tools for non-invasive cancer therapy, vaccine development and disease diagnosis. We will focus upon Ad vectors derived from serotypes 2 and 5 and belonging to subgroup C, which are the most common serotypes used for gene therapy applications because they do not cause serious illness in normal individuals and, moreover, high numbers of purified viral particles can easily be produced under good manufacturing practice standards. Descriptions of Ads will thus be limited to these two serotypes.

Adenoviral surface engineering

To enhance gene therapy efficacy and to reduce toxicity caused by immunogenic and inflammatory responses within the host, numerous strategies have been developed to target Ads to cell and tissue-specific receptors and to block interaction with the host's immune system through alteration of the viral surface. These engineering strategies, which were originally developed for gene therapy, are now being applied to conjugate several different molecules and particles to the Ad capsid, thus generating sophisticated, multifunctional nanoscale systems for diagnosing diseases, delivering therapeutic agents and monitoring treatment progression. In Table 1, existing methods for the selective assembly of various inorganic and organic molecules and particles onto the Ad capsid are outlined. Below, we will examine the application and limits of these strategies in an effort to pave the way for realization of such multifunctional nanodevices.

Genetic modification of Ad capsids

The Ad capsid consists of three major proteins, hexon, penton and fiber, and three minor capsid-stabilizing proteins, pIIIa, pVIII and pIX (Figure 1). Sites suitable

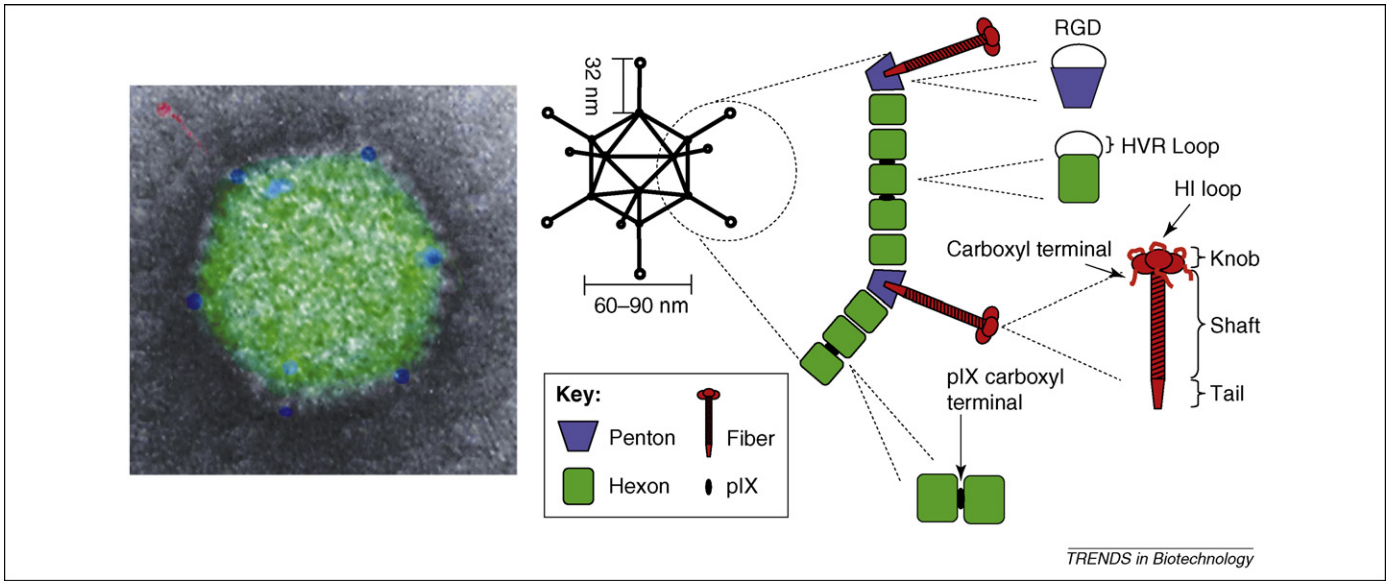

Figure 1. Representation of the adenovirus (Ad) structure. Ads have an icosahedral morphology with a core diameter of 60–90 nanometers (nm) and possess a flexible fiber trimer extending 32 nm from each of the 12 vertices. The viral capsid consists of three major proteins: hexon, penton and fiber, in addition to several proteins, such as pIX, that stabilize hexon and penton. In the left-hand picture, a false-colored electron micrograph image of an Ad is shown. Hexon is clearly visible (in green). Sites of penton are identified in blue, and a single fiber is apparent (upper-left corner, in red). The schematic illustration of the viral structure (on the right) shows the relative positions of the capsid proteins for which specific engineering strategies have been developed. The sites that are most suitable for incorporating novel peptide sequences into the capsid structure through genetic engineering are highlighted. These include the hexon hypervariable region (HVR), the penton Arg-Gly-Asp (RGD) loop, the pIX carboxyl terminal, and the fiber knob carboxyl terminal and HI loop.

Table 1. Selective incorporation of inorganic and organic molecules, as well as nanoparticles in specific sites of the Ad capsid

Macromolecule	Modification site	Engineering strategy	Refs
Peptide	Fiber knob: carboxyl terminal or HI loop	Genetic engineering	[12–14]
	Fiber knob trimer	Molecular recognition: peptide–ligand interaction	[28]
	Penton RGD loop	Genetic engineering	[9,95]
	Hexon HVR	Genetic engineering	[11,20,96]
	pIX carboxyl terminal	Genetic engineering	[15,83,97]
	Capsid surface	Self-assembly: electrostatic interactions	[84]
	Exposed capsid glutamic acid	Covalent conjugation	[44]
Protein	Fiber knob trimer	Molecular recognition: antibody–ligand interaction	[23,24]
	Fiber knob trimer	Molecular recognition: receptor–ligand interaction	[25,26]
	Penton RGD loop	Molecular recognition: antibody–ligand interaction	[9]
	Capsid surface	Self-assembly: electrostatic interactions	[85]
Photochemical or fluorophore	Exposed capsid lysines	Covalent conjugation	[55,98]
Polymer	Exposed capsid lysines	Covalent conjugation	[31,35,39,41]
Metal nanoparticle	Hexon HVR	Genetic engineering and covalent conjugation	[11]
	pIX carboxyl terminal	Genetic engineering and covalent conjugation	[11]
	Fiber knob	Molecular recognition: antibody–ligand interaction	[29]
	Exposed capsid lysines	Covalent conjugation	[43,64]
	Capsid surface	Self-assembly: electrostatic interactions	[65]
Lipid or sterol	Capsid surface	Self-assembly: electrostatic interactions	[19,87,88,94]
	Capsid surface	Self-assembly: hydrophobic interactions	[19,89–91,94]
Sugar	Exposed capsid lysines	Covalent conjugation	[30]
Radionuclide	Exposed capsid lysines	Covalent conjugation	[19,31]
Biotin	Exposed capsid lysines	Covalent conjugation	[45]
	Fiber knob carboxyl terminus	Genetic engineering and molecular recognition	[16]
	pIX carboxyl terminus	Genetic engineering and molecular recognition	[16]
	Hexon HVR	Genetic engineering and molecular recognition	[16]

for insertion of novel peptide sequences through genetic manipulation of the viral DNA encoding for different capsid regions have been identified, allowing the display of peptides at specific sites on the viral surface (Box 2).

In most cases, novel peptides inserted into the Ad capsid are designed to grant binding to alternative cellular receptors. However, peptide sequences can also be inserted to allow conjugation to antibodies [9]. In addition, novel surface chemistries can be introduced for interaction with other molecules that show high affinity for the inserted peptide sequence. For example, Kreppel *et al.* [10] introduced several short cysteine-containing peptide motifs to the fiber HI loop. This allowed for a covalent coupling of the cysteine chains to other molecules through maleimide linkage, thereby introducing a novel chemistry to the viral surface. Similarly, Saini *et al.* [11] genetically modified the Ad proteins pIX and hexon to introduce 6-histidine (6-His) residues, which allowed for binding of gold nanoparticles that contained a reactive nickel nitrilotriacetic acid (Ni-NTA) group with high non-covalent affinity for 6-His.

When incorporating peptides onto the Ad surface, several important factors need to be addressed, including limitations of peptide size, steric hindrance and potential alteration of the Ad surface charge. X-ray crystallography studies showed that the carboxyl terminus of the fiber knob domain is directed toward the Ad capsid, suggesting that any molecular conjugate also will be oriented toward the capsid [12]. By contrast, modifications at the HI loop would direct conjugates away from the capsid, thereby leaving them more accessible to cellular receptors [13]. The size limit of targeting peptides that could be attached to the HI loop was determined by Belousova *et al.* [14], who established that there is a negative correlation between increasing length of peptides and resulting viral infectivity. Similar limits have not yet been established for peptides incorporated either into the hypervariable region (HVR) or the Arg-Gly-Asp (RGD) loops of hexon and penton. Because pIX is buried between hexon trimers (Figure 1), any attached targeting ligands might not extend sufficiently from the Ad capsid and are thus likely to be inaccessible for their target receptor. To address this issue, Vellinga *et al.* [15] linked peptide spacers of ~30, 45 or 75 Å length to pIX, which allowed for greater access of targeting ligands, and found that the longest spacers were the most effective. However, vector targeting through genetic incorporation of

Box 2. Topography of Ad capsid sites suitable for genetic engineering

It is possible to introduce novel peptide sequences to specific sites on the Ad capsid through genetic engineering. Particular attention has been paid to modification of the capsid proteins hexon, penton, fiber and pIX (Figure 1), as reviewed elsewhere [6]. Each of the 720 Ad hexons contains a hypervariable region (HVR) that consists of a hydrophilic loop extending from the adenovirus capsid and that can incorporate novel peptide sequences. Likewise, the 60 pentons each possess a flexible loop extending from the Ad capsid that contains the integrin binding RGD peptide sequence, which can be replaced with other peptides. Two major sites suitable for directed mutagenesis have been identified on the fiber knob protein – the carboxyl terminus and the HI loop. The latter is a flexible and exposed region on the exterior of the knob region not involved in trimerization that connects knob β-strands H and I, which are involved in the formation of the CAR-binding site [82]. Ads have 12 fiber trimers, each with three knob domains, and therefore there are 36 potential sites for introduction of additional peptide sequences. More recently, pIX, an adenovirus structural protein, has become an increasingly important tool for modification of the Ad capsid, and peptides could be attached to the exposed carboxy termini of the 240 pIX units found on the capsid. Moreover, recent studies have demonstrated that it is also possible to delete or replace large portions of the viral DNA encoding for pIX without affecting the stability of Ad capsids [83], making pIX an attractive target for engineering strategies.

high affinity ligands was most successful when Ad fiber proteins were modified, rather than other capsid proteins. This is likely to be due to aberrant trafficking occurring at the cell surface or inside targeted the cells in cases in which binding and uptake are not fiber-mediated [16].

Care must be taken when genetically engineering Ads because the deletion of native peptide sequences or the incorporation of novel peptides could have a dramatic effect on their ζ-potential (see Glossary), thus possibly altering their biodistribution and stability in suspension [10,17–19]. At neutral pH in aqueous media, Ads possess a ζ-potential of −30 mV [19]. Incorporation of positively charged peptides, such as lysine, onto the viral capsid might shift ζ-potential towards the isoelectric point of the dispersion, leading to significant particle aggregation and greatly reducing the utility of such vectors. Additionally, Li *et al.* [17] observed that the HVR is largely hydrophilic and the strength of the surface charge accumulated on the hydrophilic and hydrophobic regions of hexon from various Ad serotypes correlated with differences in tissue tropism. Conversely, Alemany *et al.* [20] noted that deletion of an acidic stretch in the HVR decreased the negative charge of the virus but did not affect blood clearance rates. Recently, Prasuhn *et al.* [21] demonstrated that chemical and genetic modifications that modulated the surface charge of a modified bacteriophage capsid could be tailored to alter plasma clearance and tissue distribution in predictable ways. Zhang *et al.* [22] used cowpea mosaic virus and iron oxide nanoparticles to develop a theoretical model demonstrating the effects of changes in ζ-potential on cell binding and internalization of nanoparticles. These studies suggest that it is possible to manipulate the Ad ζ-potential to influence the biodistribution and cellular uptake of Ad vectors.

Ad surface engineering by self-assembly interactions

Genetic manipulation of Ads can be very time consuming and, moreover, the resulting significant structural changes can lead to the production of non-viable viruses. Therefore, several strategies for modifying the Ad capsid through self-assembly interactions have been developed. Such interactions include the use of electrostatic and hydrophobic associations (summarized in Box 3) and molecular recognition interactions for the modification of Ad surface characteristics.

Loosely defined, molecular recognition refers to the ability of one molecule to attach itself to another molecule of a complementary shape. For the modification of Ads, a molecular adaptor consisting of a capsid-binding domain, such as a fiber-directed monoclonal antibody [23,24] or the extracellular region of the coxsackie and adenovirus receptor (CAR) [25,26], is either genetically or chemically fused to a cell-binding ligand, which might include antibodies targeting cell receptors [25], growth factors [27] or peptide ligands such as gastrin-releasing peptide [28]. These adapters serve the dual purpose of blocking binding to CAR and of redirecting the virus to a new receptor. This strategy was further developed by Li *et al.* [26], who introduced an adaptable bifunctional ligand that consisted of a fusion between the extracellular domain of CAR (to confer Ad knob binding) and the Fc-binding domain of protein A. Because the Fc-binding domain will recognize any immunoglobin, this adaptor allows for conjugation of additional antibodies, even if they do not possess any affinity for Ads. The resulting vector will thus be composed of three parts: the Ad particles, the Ad knob binding ligand with an Fc domain, and the tissue-directed immunoglobin. The use of molecular recognition for Ad targeting has been extensively reviewed elsewhere [6].

The true versatility and utility of linking antibodies to the Ad capsid are only beginning to be explored. Most studies so far have focused on directing Ads to alternative receptors through the addition of new targeting ligands, but viruses can easily be conjugated to other antibody-linked materials, including synthetic components, through this type of interaction. For example, Perez *et al.* [29] conjugated Ads to nanoparticles that were composed of

Box 3. Modification of the Ad surface through electrostatic and hydrophobic interactions

Several intermolecular forces, particularly electrostatic attraction and hydrophobic interaction, allow aqueous dispersions of Ads to associate and form complexes with both charged and uncharged molecules and particles. For example, the negative ζ-potential of Ads allow them form complexes with different cationic molecules and particles, including cationic peptides [84] and proteins [85], cationic dendrimers [86], cationic liposomes [87,88] and polycation-coated metal nanoparticles particles [65]. In general, this type of modification is easily accomplished by simply mixing the virus with the cationic component and leads to electrostatically induced aggregation. However, the formation and stability of such electrostatic complexes is not easily controlled, and the exact size, shape and biological function of the resulting particle assembly will depend on a variety of factors, such as the concentration and stoichiometry of the components and the strength of the electrostatic attraction. These limitations significantly complicate the development of nanomedicines that are based on electrostatic complex formation.

The Ad capsid also possesses several hydrophobic regions in the hexon protein [17] that provide adsorption sites through hydrophobic interactions. This property allows Ads to associate with zwitterionic liposomal formulations [89,90] and free cholesterol [91]. Cholesterol seems to coat the virus, leading to the formation of small Ad aggregates. Specific links are formed between the virus and zwitterionic liposomes, most likely owing to interactions of hexon with the phosphatidylcholine headgroup. Such virus–liposome hybrid particles might present a highly promising delivery agent for liposome-encapsulated therapeutics, as the binding and uptake of Ads has been shown to enhance both endosomal escape of internalized particles [92] and uptake of membrane-associated particles [93].

We have recently demonstrated that the entire Ad capsid can be coated with cationic, neutral and polymer-conjugated phospholipid bilayers [19,94]. This was achieved by using an aqueous suspension of Ads to hydrate a desiccated phospholipid film. During this process, the lipid molecules self-assemble into bilayers on the viral surface. However, use of a high lipid to virus ratio ($\sim 10^7$ phospholipid molecules per Ad capsid) is necessary to avoid aggregation of the coated particles, and this allows for highly dispersed, stable nanoparticle suspensions [94]. In mouse models of cancer, the lipid coat was able to extend blood circulation time and reduce vector immunogenicity and allowed for tumor-specific transduction via the systemic circulation in the absence of high levels of gene transfer to other tissue [19], thereby providing the basis for the development of a novel vector platform for the systemic delivery of adenovirus to disseminated targets. Moreover, the general approach of using lipid coating to modulate the biological function of non-enveloped viruses, such as Ads, might have wide-reaching implications that are broader than gene therapy.

an iron oxide core caged with a dextran coating, onto which Ad-specific antibodies were attached. This interaction generated supramolecular assemblies consisting of Ad particles that were crosslinked to multiple other Ad particles through metallic nanoparticle bridges [29]. The resulting material might have uses in applications that take advantage of changes in the magnetic properties of the metallic nanoparticles that occur upon viral-induced assembly, thereby allowing their use as nanosensors for rapid and sensitive clinical detection of Ads [29]. Such supramolecular assemblies might also have broader implications for the development of nanoscale electronics.

Ad engineering through conjugation chemistry

The Ad capsid possesses ~1800 free lysines, the majority of which are located on hexon, penton and the fiber proteins [30]. These free lysines can be covalently linked to other molecules, such as polymers, sugars, biotin and fluorophores, or even, via amide bonds, to metal nanoparticles and quantum dots (Table 1). As discussed above for genetic capsid modifications, polymers or other molecules covalently conjugated to the Ad capsid can have a dramatic effect on the physicochemistry and pharmacokinetics of Ads, altering size and ζ- potential, as well as affecting blood clearance rates. Therefore, these characteristics need to be critically assessed when any such modification is introduced.

Polymer coating of the viral surface has been applied with the aim of reducing the interaction of the virus with blood proteins and its clearance by blood and tissue macrophages, thereby leading to increased vector persistence in the blood [20,31]. Further, the resulting vectors were resistant to neutralizing antibodies [32,33], were able to reduce the innate immune response directed against the vector [34] and possessed reduced CAR tropism [35] while continuing to effectively transduce target tissue [36,37]. The most commonly studied polymer, monomethoxy-polyethylene glycol (mPEG), can be bound to the N terminus of free lysine groups on all three Ad proteins, hexon, penton and fiber, and results in PEG polymers that extend from sites throughout the entire Ad capsid [32–37]. Although PEG was able to ablate CAR-binding *in vitro*, substantial alteration of *in vivo* uptake has not yet been achieved. However, Len Seymour's group [38] recently showed that an Ad vector, in which the polymer hydroxylpropyl methacrylamide (pHPMA) was covalently bound to capsid proteins, demonstrated long circulation and, furthermore, passively accumulated in and efficiently transfected solid tumors after intravenous injection into mice. Each pHPMA strand makes multiple bonds with the Ad capsid with an average of 14 points of attachment, which leads to an increase in the measured particle diameter from 105 nm to ~128 nm [39]. However, increased vector size due to polymer coating might also reduce dissemination into tumors. This has been demonstrated for Ad particles that had been coated with poly(methylacrylic acid-(dimethylamino)-ethyl)-methyl-amino-ethyl ester (pDAMA) and that did not diffuse *in vitro* into the center of three-dimensional tumor spheroids owing to their increase in size [40]. Enhanced polymer modification of Ads can be achieved with the use of bifunctional polymers, which are attached to the virus at one end and to other molecules, including protein ligands like fibroblast growth factor or phage-selected targeting peptides, at the other end [37,41].

Using the same lysine-binding sites used for polymer coating, Pearce *et al.* [30] linked the sugars galactose and mannose to the Ad surface, which allowed the virus to target sugar-specific receptors. Dynamic light scattering analysis of the resulting vectors indicated an increase in the measured mean hydrodynamic diameter from 120 nm before glycosylation to 200 nm after glycosylation. Because electron micrographs did not show an increase in vector size, the increase in the diameter measured by light scattering was likely to be caused by the presence of sugars bound to lysine on the ends of the fiber. Because lysine residues on the Ad fiber knob are required for interaction with CAR [42], glycosylation also decreased the transfection ability of Ads through CAR. Conjugation of any other moiety to this region might also inhibit Ad binding to CAR.

This might explain the observation by Everts *et al.* [43], who conjugated gold nanoparticles to lysines at the Ad surface and noted that at a high ratio between nanoparticles and Ad particles, Ad-mediated transduction was greatly reduced, possibly owing to extensive binding of the nanoparticles to fiber knob lysines. To circumvent this problem, the authors genetically modified the capsid proteins pIX and hexon to introduce 6-His sites [11], which allowed the coupling of commercially available gold nanoparticles that contained a reactive Ni-NTA group with high non-covalent affinity for 6-His. The site-specific tethering of these nanoparticles prevented interference with Ad binding to CAR and integrin owing to nanoparticle attachment to lysines on fiber and penton. Electron microscopy showed that the virus capsid remained intact after decoration with nanoparticles [11,43], but detailed physicochemical analysis will need to be conducted to fully understand the surface properties of the hybrid particles.

Using a different surface chemistry, Turunen *et al.* [44] demonstrated that, with the help of transglutaminases, peptides could be linked to glutamic acid residues that are present on the Ad surface. Another functional group could be introduced onto the capsid surface through covalent binding of the small biotin molecule to the Ad proteins hexon, penton and fiber [45]. As an alternative approach, Campos *et al.* [16] introduced biotin acceptor peptides (BAPs) to the carboxyl terminus of the fiber knob domain, the carboxyl terminus of pIX and the hexon HVR loop by site-directed mutagenesis, and these sites were subsequently biotinylated during virus production. Addition of biotin allows for conjugation with avidin, which upon reaction with biotin forms one of the strongest known non-covalent bonds [46]. This approach has been successfully used to conjugate biotinylated Ad particles to peptides, proteins, polymers and antibodies that had been fused to avidin [45,47].

Adenoviruses for engineering of multimodal therapeutic and imaging agents

Surface-modified Ads could be considered the ideal vehicle for several emerging applications, including non-invasive cancer therapy, vaccine development and imaging, which all rely on highly localized targeting. The ability to link

multiple functionalities to the Ad capsid theoretically allows the incorporation of several therapeutic and diagnostic modalities within a single vector, although such an Ad-based vector has not yet been developed. Furthermore, Ads have the capacity to anchor entire nanoparticles to their surface. Many of the properties that are associated with metal nanoparticles are dependent upon their size and shape [48]. Therefore, anchoring them to a larger carrier such as Ad particles might not only prevent their aggregation and enhance activity but might also alleviate fears over the safe use of nanoparticles, which are considered to pass unhindered through normal cellular barriers. Such Ad-carrier-based nanoparticles would be greater than 5 nm, thus avoiding renal filtration, but significantly smaller than 300 nm, thus ensuring efficient internalization into the target cell. In addition, the Ad scaffold would degrade into non-toxic and excretable by-products and would possibly carry the attached nanoparticles with it out of the cell. The high uniformity with regard to dimension and surface chemistry of these Ad-linked nanoparticles would also improve their chance of being accepted by the U.S. Food and Drug Administration (FDA) and corresponding agencies throughout the world. A detailed discussion of their current applications and future possibilities is presented below.

Ad-based nanoparticles as cancer therapeutics

Ideally, the goal of any cancer therapy is to selectively target and destroy diseased tissue while sparing the surrounding healthy tissue. Ad particles have been investigated for use in cancer gene therapy and oncolytic viral therapy, as discussed extensively elsewhere [49,50]. Here, the focus will be on the use of Ad particles as carriers for effectors, which are able to enhance non-invasive cancer therapeutics, such as radiation therapy, photodynamic therapy (PDT) and photothermal therapy (PTT) (see Glossary).

What makes Ads particularly attractive as carriers for sensitizers of cancer cells to non-invasive therapies is that Ads themselves act as sensitizers through their natural functions. Ads produce a linear, double-stranded viral genome that does not integrate with the genome of the host cell during replication but induces a DNA double-strand break (DSB) repair response in the host cell [51,52]. Expression of the adenoviral E4orf6 protein represses the ability of the host cell to repair DNA DSBs [52]. Thus, by inhibiting the capability of infected cells to repair damaged host cell DNA, the Ad sensitizes the host cell for concurrent radiation damage [51]. Conversely, delivery of ionizing radiation to the tumor site creates an environment that is more conducive to Ad replication, which might enhance the activity of oncolytic adenoviral therapy [53]. Furthermore, expression of the adenoviral gene E1A can sensitize infected cells to the cytotoxic effects of oxidative stress and enhance the accumulation of reactive oxygen by impeding the cellular response to oxidative stress [54]. Therefore, therapeutic strategies that induce DNA DSBs and cause oxidative stress might act synergistically with Ad infection.

Nearly ten years ago, it was shown that Ad proteins could be linked to the organic photosensitizer phthalocyanine (Pc) to deliver the molecule to cancer cells both *in vitro* and *in vivo*, which made these cells responsive to PDT [55]. Conceivably, Pc also could be conjugated to an intact Ad capsid, which would make the vector-infected cells even more sensitive to PDT due to inhibition of the DNA DSB repair pathway and of oxidative stress responses and which would allow for additional modifications of the Ad surface to enhance tumor targeting. In addition to photosensitizers, heavy metals have also been used to enhance the effectiveness of ionizing radiation doses delivered in radiation therapy of cancer [56]. In a breakthrough study, it was shown that intravenously delivered gold nanoparticles, in combination with ionizing radiation, were able to eliminate cancer in 86% of tumor bearing mice, in contrast to a 20% cure rate achieved with radiation therapy alone [57]. However, if gold nanoparticles were conjugated to the surface of Ads, they could be directly targeted to tumors, resulting in a potential improvement in cancer elimination. Furthermore, after cellular uptake, Ads escape the endosome, in which naked metal particles tend to accumulate [58], and travel to the nuclear envelope, where they bind to the nuclear pore complex [59]. The electrons that are generated by the interaction of ionizing radiation with metal nanoparticles possess extremely short path lengths in cells, and the close proximity to the nucleus should thus increase the capacity of such particles to cause DNA damage. The rate of degradation and elimination of metal nanoparticles *in vivo* is not yet known, but their conjugation to Ad particles should facilitate elimination of the tethered nanoparticle, which might also help to overcome the current uncertainty over the toxicity of nanomaterials.

Metallic nanoparticles also have been used as photosensitizers for PTT [60], and consequently gold-nanoparticle-labeled Ad particles could allow the combination of gene or oncolytic viral therapy with PTT cancer therapy [43]. The aggregation of photosensitizers is a common problem that can prevent the generation of heat and therefore affect the outcome of therapy [61]; this could be prevented by attaching the photosensitizers to a viral particle. Another strategy for generating localized heat is the use of paramagnetic metal nanoparticles that are placed into an oscillating electromagnetic field [62,63]. Ads have been successfully conjugated to paramagnetic nanoparticles [29,64,65], and although the resulting particle was not tested for generation of heat, its application for PTT is conceivable.

Because of its rapid blood clearance, hepatic tropism and potential for inflammatory toxicity, adenoviral-mediated cancer therapy so far has been limited to local delivery in clinical trials. However, coating Ad particles in polymers or lipids was shown to reduce inflammatory and immune responses and increased their blood circulation time in mice, which allowed for effective targeting to tumor sites [19,31,38]. Moreover, a high level of viral gene expression was detected in tumors that were established in the flanks of mice after systemic injection of both lipid- and polymer-coated Ad particles. Strikingly, hepatic gene transfer was reduced up to 1000-fold. Overcoming the native hepatic tropism of Ads is a major hurdle towards providing site-specific delivery of Ad-based therapeutics.

Recently, the mechanism by which Ads infect hepatocytes was discovered and was found to involve the binding of blood coagulation factor X to Ad hexon HVRs, which is followed by hepatocyte uptake of the complex, mediated by a heparin-binding site in the factor X serine protease domain [66]. This discovery, combined with the above findings, might help to greatly reduce the inflammatory toxicity and off-target uptake of intravenously injected Ads, which currently limits their dose. Based on the presented strategies, an idealized vector for cancer therapy could be proposed, as shown in Figure 2.

Ad-based vaccine nanoparticles

Although the development of vaccines can be considered one of the greatest achievements of modern medicine, there is still not an effective vaccine for most human or animal diseases. It is crucial that new strategies for vaccine production are developed, and the application of Ads for use as a genetic vaccine is well established in animal models [67,68], although no clinically relevant vaccine has yet been produced. Because administration of Ads generates a strong immune response, which might enhance the response to other antigens co-delivered with Ads, the Ad capsid recently has been employed to display antigenic peptides for the development of vaccines against influenza and *Pseudomonas aeruginosa* [69,70]. Such vectors were shown to be effective at inducing immune responses against peptides incorporated on several sites on the viral capsid, including the hexon, penton, fiber knob and pIX. Surprisingly, peptides displayed from the fiber knob domain induced the strongest immune response [70]. In this case, a maximum of 36 copies of the antigenic peptide could be displayed, as opposed to display of 720 copies arising from hexon HVR modification, indicating that the choice of presentation site, rather than the number of peptides displayed, is crucial for potential use as a vaccine. This observation also implies that incorporation of any peptide needs to be carefully considered to avoid undesired immune responses when used for purposes other than vaccination. Vaccine strategies could be further enhanced by targeting and activation of dendritic cells (DCs) through interactions with CD40 receptors [71]. The covalent incorporation of the sugar mannan onto the Ad capsid was shown to lead to particle uptake by DCs and enhanced the tumor vaccine action of Ads carrying an immunogenic transgene [72]. The authors of this study suggested that the increased transfer of the antigen to DCs in the presence of mannan could be a possible explanation for the improved anti-tumor effects. These observations provide further insights into the design of novel vaccine strategies and might be relevant for future applications of antigens identified for other diseases, and an idealized vaccine vector is shown in Figure 3.

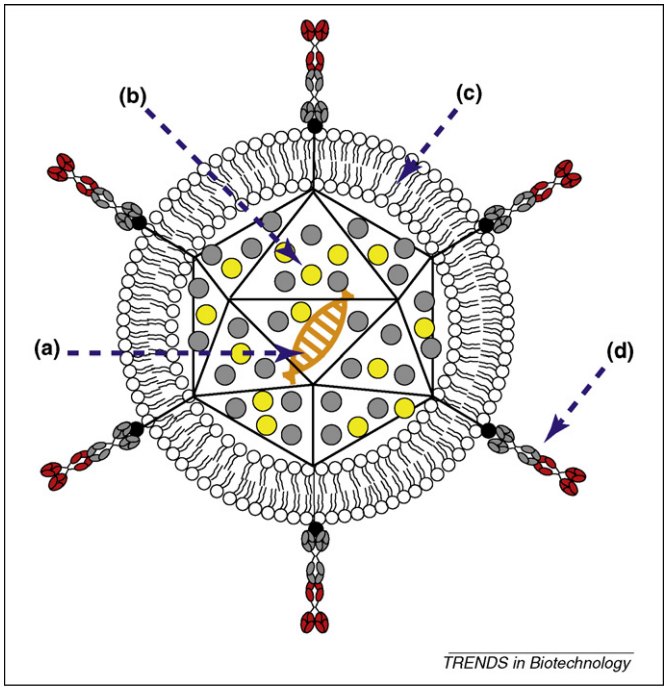

Figure 2. Idealized Ad-based nanoparticle for cancer therapy. The nanoparticle shown here incorporates several functionalities that are based on different engineering strategies. **(a)** Genetic engineering: a replication-competent virus encodes E1A and E4orf6 proteins (symbolized by orange DNA), which allow for oncolytic viral therapy [50] and for sensitization to chemotherapy or radiation therapy [51,52,54]. **(b)** Decoration of the viral surface with metal nanoparticles: covalent conjugation of metal nanoparticles to the viral surface allows for MRI or CT imaging, photothermal therapy (PTT) and dose enhancement of radiation therapy. Several types of metal particle could be incorporated simultaneously, such as gold nanoparticles for CT, PTT and radiation therapy and iron oxide nanoparticles to allow simultaneous MRI imaging. **(c)** Virus coating with lipid bilayers: coating of the Ad particle with self-assembled lipid bilayers might block its interaction with blood or the mononuclear phagocyte system without altering the viral surface, which remains available for conjugation with nanoparticles or other molecules. Lipophilic chemotherapeutics could also be incorporated into the bilayer, increasing the functionality of the resulting particle. **(d)** Modifying Ad fibers with targeting ligands. Shown here is the addition of a dual binding ligand that contains a fiber-binding antibody on one end (in gray) to block interactions between the fiber and is native cellular receptor. It is fused to another antibody fragment (in red) directed against a tumor-specific receptor or antigen to aid tumor targeting and uptake.

Ad-based nanodiagnostic imaging agents

Conventional imaging techniques, such as computed tomography (CT) and magnetic resonance imaging (MRI), can be used to monitor abnormalities associated with cancer. In particular, early detection of the spread of malignant cells to the lymph nodes is key to planning treatment [73]. Recently, an MRI contrast agent based on iron oxide paramagnetic nanoparticles was developed that was able to help identify tumor-infiltrated lymph nodes in individuals with prostate cancer [74]. However, to identify the metastatic region, this method relied on macrophages engulfing the magnetic particles and then infiltrating the node. The low sensitivity of this agent, which arises from the need for engulfment of the magnetic particles and their accumulation in the lymph node, might be overcome with the use of nanoparticle-labeled Ad particles. The distribution of nanoparticles in the lymphatic system is known to correlate with the size and surface charge of the particles [75], and the negatively charged Ad capsid and its size will favor the entry of Ad particles into the lymphatic system. In agreement with this hypothesis, it was recently demonstrated that adenoviral vectors can effectively function as lymphotropic agents. They were able to travel through the lymphatic vessels to mediate gene transfer into metastatic prostate cancer cells in the draining lymph nodes after injection into a primary tumor in mouse models [76].

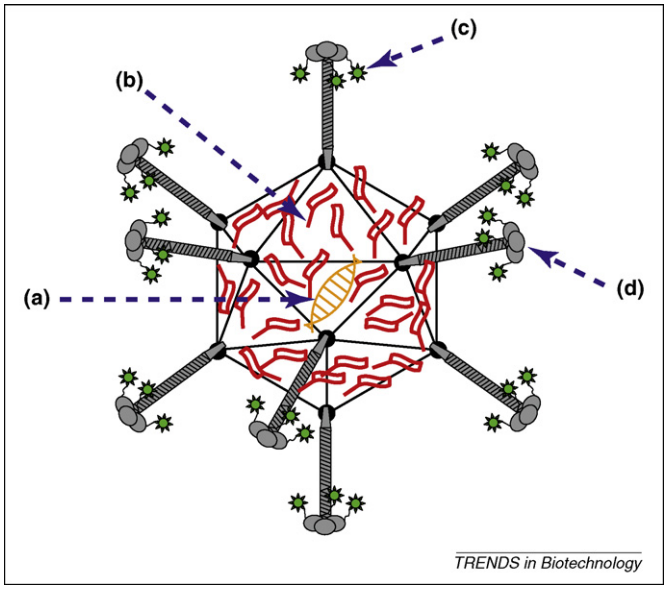

Figure 3. Idealized Ad-based vaccine nanoparticle. The nanoparticle shown here incorporates several functionalities that are based on different engineering strategies. **(a)** Genetic engineering: a replication-deficient viral genome encodes for an antigenic protein and a protein able to activate immune cells. **(b)** Carbohydrate-coating of exposed lysine residues on the Ad capsid: the Ad surface is covalently conjugated to carbohydrates (red flags), such as sugars or an antigenic polysaccharide derived from a bacterial cell wall, which act as a vaccine adjuvant and can aid in uptake into antigen presenting cells (APCs), as well as possibly blocking the interaction of virus particles with blood proteins. **(c)** Display of antigenic peptides: antigenic peptides (indicated by green stars) can be conjugated to the carboxyl terminal of the fiber knob domain, which was previously shown to be the site on the viral surface with the highest immunogenicity. **(d)** Fiber knob targeted to an APC: use of a peptide motif to bind receptors such as CD40 on APCs might not only enhance uptake of the Ad vector but could also lead to activation of the APC, thus further enhancing the action of the vaccine.

Surface modification of Ads also allowed the incorporation of additional imaging and contrast agents, which were used to detect the involvement of lymph nodes in cancer progression with high sensitivity [77]. Paramagnetic particles, which allow for MRI detection [64], and radionuclides [19,31], which permit imaging by positron emission tomography (PET) or using a γ-camera, have been successfully conjugated to Ad capsids without loss of function. This type of surface decoration is not just an exercise in nanoparticle engineering. Several studies have employed the outer coat of viruses with the aim of incorporating diagnostic imaging agents, including the plant viruses cowpea mosaic virus and cowpea chlorotic mottle virus, as well as several bacteriophages (reviewed in [1]). Measurements with typical clinical MRI equipment showed that MR signals for viruses coated with contrast agent were up to 1000-fold stronger than those produced by any currently used MR contrast agent [78]. Furthermore, Burton *et al.* [79] reported that Ad-mediated gene delivery of a PET imaging agent allowed the identification of lymph node metastasis in a mouse bearing 2.5-mm lymph node lesions, a decisive improvement over the current clinical lymphotropic MRI method, which is only able to detect lesions of at least 5–10 mm in diameter.

Concluding remarks

Engineering of nanoscale biomedical delivery systems has grown into its own cross-disciplinary field, capturing the interest of both academic and industrial researchers. The bottleneck in the clinical development of novel therapeutics using any nanomaterials is the current lack of relevant structural and physicochemical characterizations and knowledge of biological function relationships at the pre-clinical level, which would help to interlink cell culture, mouse and human studies. This barrier might be overcome with the use of viral delivery systems. Previous studies of the structural and molecular basis of viruses as pathogens and their development for use as targeted gene delivery vehicles now allows viruses to be used as tools for biomedical nanotechnology. There are no other nanoparticle platforms that can achieve the same degree of control over size, homogeneity and versatility as the molecular shuttles offered by viral vectors. A detailed understanding of their capsid structure and surface chemistry makes Ads an excellent starting point. Because of the availability of numerous modification sites on the Ad capsid, future vectors might be developed that incorporate several functionalities discussed herein, making them excellent candidates for a combined delivery of cancer therapeutics, vaccines and diagnostic and imaging agents. However, the addition of any new functionality must be carefully balanced with the retention of the desired Ad characteristics and functions to maximize the potential of such delivery systems. Ideally, Ad-based nanocarriers could be used as multifunctional entities able to diagnose disease, deliver therapeutic agents and monitor treatment progression. With future improvements in vector design, nanomedicine might finally be able to live up to its potential.

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