

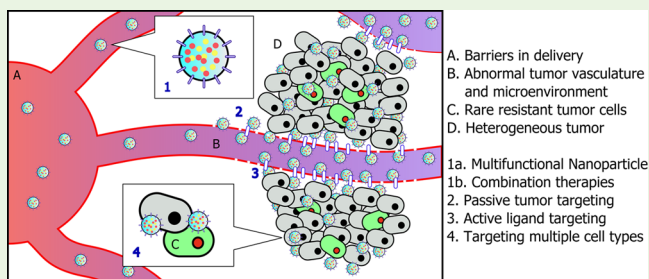
# Nanotherapy for Cancer: Targeting and Multifunctionality in the Future of Cancer Therapies

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**ABSTRACT:** Cancer continues to be a prevalent and lethal disease, despite advances in tumor biology research and chemotherapy development. Major obstacles in cancer treatment arise from tumor heterogeneity, drug resistance, and systemic toxicities. Nanoscale delivery systems, or nanotherapies, are increasing in importance as vehicles for antineoplastic agents because of their potential for targeting and multifunctionality. We discuss the current field of cancer therapy and potential strategies for addressing obstacles in cancer treatment with nanotherapies. Specifically, we review the strategies for rationally designing nanoparticles for targeted, multimodal delivery of therapeutic agents.

**KEYWORDS:** nanoparticle, gene delivery, drug delivery, cancer therapy



## INTRODUCTION

**History of the Field of Cancer Therapy.** The first documented case of cancer was recorded in Egypt around 3000 BC, and surgical resections were historically the predominant mode of treatment.<sup>1</sup> Emile Grubbe began using X-rays to treat recurrent breast carcinoma in 1896, and surgery and radiotherapy continued to be the mainstay of treatment. It was not until 1948, when Sidney Farber evaluated the use of antifolate compounds for treating leukemia in children, that chemotherapy became a viable treatment option.<sup>2,3</sup> The ensuing decades have generated numerous advances in cancer biology and chemotherapy. The evaluation of the biochemical processes involved in drug resistance advanced in the 1960s, and resulted in the implementation of combination chemotherapies in 1965 and adjuvant chemotherapy in 1972.<sup>3</sup> Significant improvements in cancer mortality were first noticeable in the 1990s, and the field of targeted cancer therapies blossomed with new discoveries in cancer signaling pathways involved in tumor development, proliferation, and metastasis.

The completion of the Human Genome Project in 2003 created the potential for revolutionary advances in understanding many human diseases including cancer. Multiple international projects, like The Cancer Genome Atlas, were initiated to analyze the genetics of multiple cancer subtypes. Through extensive genome analysis of tissues from several patients, researchers are gaining a stronger understanding of the development, susceptibilities, and prognosis of individual cancers, and in turn learning how to design patient specific therapies. Consequently, the death rates for common cancers, such as prostate, breast, lung, and colorectal, are declining because of the development of new small molecules and immunotherapies, target specific screens, and new combination

therapies.<sup>4</sup> In 2013, the five year survival for cancers from all sites has increased to 66.7%. Despite these promising advances, cancer is the second leading cause of death in the United States, with more than 1.6 million new cases annually, and approximately 580 000 deaths each year.<sup>5</sup> These numbers will likely increase with the expected aging of the population. Even as cancer survivors live longer, they are at a higher risk for developing new malignancies. In addition, the issue of drug resistance and tumor recurrence continue to hinder cancer therapies.

**Cancer Biology and Rational Treatment Design.** The birth of scientific oncology ensued when Rudolf Virchow examined blood samples from leukemia patients under the microscope in 1847. Currently, cancer pathogenesis is considered a complex multistep process where cells attain certain hallmark properties as a result of both genetic and epigenetic alterations.<sup>6,7</sup> Carcinogenesis is primarily a consequence of changes in the genetic code or gene expression. The affected genes can be categorized into three main groups: oncogenes, tumor suppressor genes, and mismatch-repair genes. Changes in gene expression may in turn allow cells to maintain proliferative signaling, evade growth suppressive signals, resist cell death, promote invasion and metastasis, confer replicative immortality, deregulate cellular energetics, promote genomic instability, and initiate angiogenesis.<sup>8</sup> Additionally, tumor interactions with adjacent stroma and the immune system can promote proliferation and metastasis through avoiding immune destruction and stimulating tumori-

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Table 1. Hallmarks of Cancer Pathogenesis and Therapeutic Implications<sup>a</sup>

hallmark of cancer pathogenesis <sup>6</sup>	cellular and molecular alterations <sup>6,13,14</sup>	potential targeted therapies
1. sustaining proliferative signaling	↑ MAP-kinase pathway, ↑ PI3K pathway, ↓ PTEN, mTOR kinase pathway	tyrosine kinase inhibitors, <sup>15</sup> proteasome inhibitors, <sup>16</sup> mTOR inhibitors, <sup>17</sup> PI3K inhibitors, <sup>18,19</sup> HDAC inhibitors <sup>20</sup>
2. evading growth suppressors	↓ TP53, ↓ RB, ↓ NF2, ↓ LKB1, TGF- $\beta$ signaling	cyclin-dependent kinase inhibitors <sup>21</sup>
3. avoiding immune destruction	↓ CTLs, ↓ CD4+ Th1 cells, ↓ NK cells, ↓ PD-1 signaling, ↑ Tregs, ↑ MDSCs, TGF- $\beta$ signaling	cancer vaccines, <sup>22</sup> ex vivo T cell modifications, <sup>23</sup> immune activating anti-CTLA4 mAb, <sup>24</sup> PD-1 agonists <sup>25</sup>
4. enabling replicative immortality	↑ telomerase, ↓ TP53	telomerase inhibitors <sup>26</sup>
5. tumor promoting inflammation	B lymphocytes, macrophages, mast cells, myeloids progenitors, necrosis, neutrophils, T lymphocytes, ↑ IL-1 $\alpha$ , ↑ reactive oxygen species	anti-inflammatory drugs <sup>27</sup>
6. activating invasion and metastasis	↑ CCL5/RANTES, ↑ c-Met, ↑ CSF1, ↑ CCPs, ↑ heparanase, ↑ EMT, ↑ IL-4, ↑ matrix-degrading enzymes, ↑ N-cadherin, ↑ Wnt signaling, ↓ E-cadherin, snail, slug, TGF- $\beta$ signaling, twist, Zeb1/2, macrophages, neoplastic stroma	inhibitors of HGF/c-Met <sup>28</sup>
7. inducing angiogenesis	↑ FGF family proteins, ↑ Ras, ↑ Myc, ↑ VEGFa, ↓ endostatin, ↓ plasmin, TGF- $\beta$ signaling, ↓ TSP-1, endothelial cells	angiogenesis inhibitors <sup>29</sup>
8. genome instability and mutation	↓ BRCA, ↓ TP53	PARP inhibitors <sup>30</sup>
9. resisting cell death	↑ A1, ↑ Bcl-2, ↑ Bcl-xL, ↑ Bcl-w, ↑ Mcl-1, ↑ extrinsic growth factor signaling, ↓ Bax, ↓ Bak, ↓ BH3 proteins, ↓ TP53, ↓ extrinsic ligand-induced death pathways	proapoptotic BH3 mimetics, <sup>31</sup> Bcl-2 antagonists, <sup>32</sup> PARA therapy <sup>33,34</sup>
10. deregulating cellular energetics	↑ GLUT1, ↑ HIF, ↑ IDH1/2	aerobic glycolysis inhibitors <sup>35</sup>
11. deregulating autophagy	Beclin	autophagy inhibitors <sup>13,36</sup>
12. tumor microenvironment	cancer stem cells, endothelial cells (notch, neuropilin, Robo, and Eph-A/B signaling), fibroblasts, myofibroblasts, neoplastic stroma, pericytes, TGF- $\beta$ signaling	antistem cell antibodies, <sup>37</sup> PDGF receptor inhibition <sup>38</sup>

<sup>a</sup>Bax, Bcl-2-associated X; Bcl, B-cell lymphoma; BRCA, breast cancer; CCL5/RANTES, Chemokine (C–C motif) ligand 5/regulated on activation, normal T cell expressed and secreted, CCPs, cysteine cathepsin proteases; CD4, cluster of differentiation 4; CSF, colony-stimulating factor; CTL, CD8+ cytotoxic T lymphocytes; EMT, epithelial-mesenchymal transition; EPH, ephrin type; FGF, fibroblast growth factor; GLUT, glucose transporter; HDAC, histone deacetylase; HGF, hepatocyte growth factor; HIF, hypoxia-inducible factor; IDH, isocitrate dehydrogenase; IL, interleukin; LKB1, liver kinase B1; MAP, mitogen-activated protein; MCL, myeloid cell leukemia; MDSCs, myeloid-derived suppressor cells; mTOR, mammalian target of rapamycin; NF2, neurofibromin 2 (merlin); NK, natural killer; PARA, proapoptotic receptor agonist; PD-1, programmed death-1; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3 kinase; PTEN, phosphatase and tensin homologue; RB, retinoblastoma; Robo, roundabout; TGF, transforming growth factor; T<sub>H</sub>, T helper; Tregs, regulatory T cells; TSP, thrombospondin; VEGF, vascular endothelial growth factor; ZEB, zinc finger E-box-binding homeobox.

genic inflammation. Epigenetic factors can also promote carcinogenesis without directly conferring any genotypic variations. Deoxyribonucleic acid (DNA) methylation, histone modification, and gene silencing are involved in cancer pathogenesis.<sup>9–12</sup> The multiple, interconnected pathways complicates efforts for providing effective therapies. Therefore, a paradigm shift is underway as researchers are working to analyze individual tumors in order to design therapies for specific cancer phenotypes.

Understanding cancer pathogenesis has allowed for development of more effective therapies. For instance, cancer cells can receive increased proliferative signaling by up-regulating surface growth factor receptors such as EGFR. These discoveries have been translated into promising clinical therapies in the form of EGFR specific inhibitors. Similarly, by analyzing specific hallmarks necessary for cancer progression, new pipelines of therapeutics are being developed to treat the disease. Table 1 describes the multiple hallmarks of cancer pathogenesis, respective cellular and molecular alterations, and associated targeted therapies.<sup>6,13,14</sup> Molecular and genetic analysis allows physicians to detect, classify, monitor, and treat cancer more effectively. However, designing adequate therapies is difficult because of the intricacies of cancer biology and the vast heterogeneity of tumors.

**Obstacles in Cancer Chemotherapy.** Although our understanding of cancer pathogenesis is increasing, the disease process remains extremely complex and much is still unknown.

Every cell lineage in the body can be affected. Inherent genomic instability and biological diversity in cancer cells can lead to treatment resistance. Only a small fraction of tumor cells is highly sensitive to therapy, and even those cells can develop resistance and progress into a more aggressive disease. Drug resistance can be either intrinsic or acquired. Intrinsic resistance occurs when tumor cells have decreased or no sensitivity to a therapeutic agent. Cells can develop resistance through a variety of mechanisms including decreased drug uptake, up-regulation of drug efflux transporters, aberrant cell cycle checkpoints, increased DNA repair, increased drug metabolism, induction of stress response genes, and inhibition of apoptosis.<sup>39</sup> Additionally, an individual cell can become resistant through its own unique variation, which further complicates cancer therapies. Acquired resistance occurs when neoplasms that were initially responsive to certain therapies becoming unresponsive. Similar to the development of antibiotic resistance in bacteria, use of chemotherapeutic agents can lead to selection for inherently resistant tumor cells. The characteristic genomic instability of many cancer subtypes and the mutagenic properties of chemotherapeutic interventions lead to new mutations that translate into drug resistance. Of note, hierarchies in signaling cascades involved in tumor development can diminish efficacy of targeted therapies if the new mutation overrides the targeted factor in the signaling cascade. For instance, mutations in the tumor suppressor gene, PTEN, can decrease efficacy of anti-HER2 immunotherapy, or

Table 2. Examples of Nanoparticle Therapeutics<sup>a,63,64</sup>

nanocarrier	name	formulation	indication	status
inorganic nanoparticle	Ferumoxide <sup>65</sup>	iron oxide MRI contrast agent	liver imaging	approved 1997
	CYT-6091 <sup>66</sup>	TNF $\alpha$ -PEG-gold	solid tumors	phase I
liposome	Doxil <sup>67</sup>	liposomal doxorubicin	ovarian, breast cancer	approved 1995
micelle	NKTR-102 <sup>68</sup>	PEG-micelle Irinotecan	colorectal and breast cancer	phase III
protein nanoparticle	Abbraxane <sup>69</sup>	paclitaxel-albumin	metastatic breast cancer	approved 2005
polymeric micelle	Genexol-PM <sup>70</sup>	micellar paclitaxel	breast, lung, pancreatic cancer	phase II–IV
polymer-drug conjugate	Xyotax <sup>71</sup>	paclitaxel-poly-L-glutamic acid	breast, ovarian cancer	phase III
	Oncaspar <sup>72</sup>	PEG-L-asparaginase	acute lymphoblastic leukemia	approved 2006
polymer nanoparticle	BIND-014 <sup>73</sup>	docetaxel-PLGA/PLA-PEG with targeting ligand	nonsmall cell lung cancer, prostate cancer	phase II
radio-immunoconjugate	Zevalin <sup>74</sup>	anti-CD20 conjugated to yttrium-90 or indium-111	non-Hodgkin's lymphoma	approved 2002

<sup>a</sup>PEG – polyethylene glycol; PLA, polylactic acid; PLGA, poly(lactic-co-glycolic) acid; TNF, tumor necrosis factor.

inactivation of TP53 can minimize the cytotoxicity of several cancer therapies.<sup>40</sup> Mutations and natural selection are fundamental in the development of resistance, as they are the primary drivers of cancer pathogenesis.

The two overarching models of carcinogenesis are the stochastic clonal evolution model and the hierarchal cancer stem cell model.<sup>41</sup> The original stochastic model describes tumor pathogenesis as the progression of somatic mutations that lead to isolation of a dominant cancerous clone that, through selective influences, eventually progresses into metastatic tumors. However, during the past two decades, studies have shown that certain cancer types arise from a more hierarchal organization of cells. These tumors are comprised of multiple cell subpopulations having a spectrum of proliferative and regenerative capabilities. This phenomenon was first described in human acute myeloid leukemia cells,<sup>42</sup> by isolation of rare leukemia initiating cells that have differentiation and proliferative capabilities similar to leukemic stem cells, and demonstration that these cells are responsible for the regenerative, self-perpetuating, and diverse nature of the tumor. The primitive tumor initiating cells are inherently quiescent and less susceptible to traditional agents that target rapidly proliferating tumor cells.<sup>41,43</sup> Subsequent studies have identified tumor initiating cells in breast cancer,<sup>44,45</sup> colon cancer,<sup>46–48</sup> melanoma,<sup>49</sup> and brain tumors.<sup>50–52</sup> Therefore, effective therapies will require targeting of these rare initiating cells since traditional chemotherapies target only the proliferative subset of the cancer.

In addition to the challenges presented by tumor cell biology, the tumor microenvironment and host factors can influence efficacy of current chemotherapy regimens. The tumor microenvironment, including both the abnormal vasculature and adjacent stromal cells, can impede drug delivery or increase drug clearance. Delivery to the tumor can be impaired in large, necrotic malignancies. Host factors, including decreased absorption, rapid metabolism, and increased clearance of agents, can lower serum drug concentrations. Additionally, drug solubility and size can hinder tumor delivery and penetration. Patients also have variable capacities to tolerate chemotherapy agents, and the development of side effects can significantly obstruct dosing and treatment duration.<sup>39</sup>

Because of tumor heterogeneity and the development of drug resistance, future therapeutic regimens will likely incorporate combination multimodal therapies. Mathematical models can be used to evaluate tumor response to targeted monotherapy and combination therapies.<sup>53</sup> These models suggest that dual therapy is often adequate for long-term disease control, but that patients with a larger initial disease burden may require triple

therapy. Further, the models suggest that simultaneous therapy is more effective than sequential therapy.

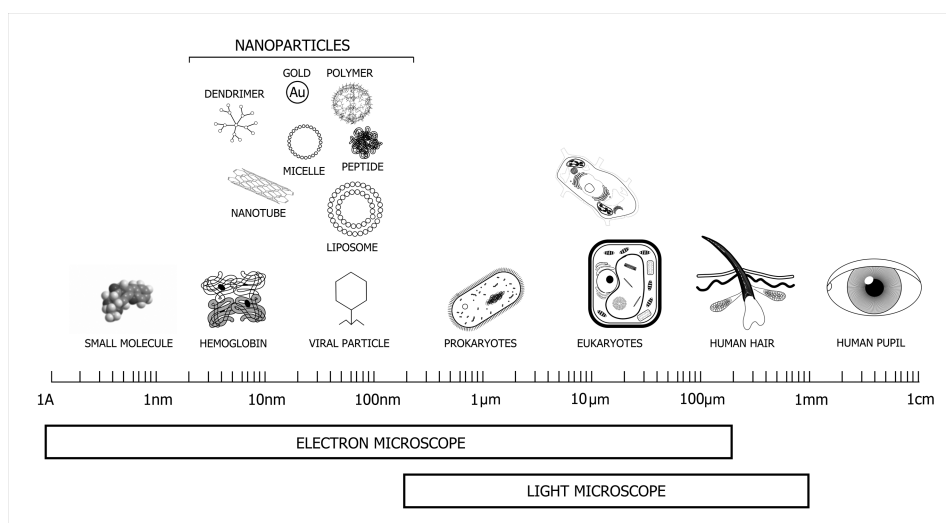
**Cancer Genetics, Tumor Profiling, and Personalized Medicine.** With the discovery of numerous clinically relevant cancer genes, gene editing is becoming an increasingly relevant aspect of cancer therapy. Gene editing via RNA interference (RNAi), through small interfering RNAs (siRNA) or micro-RNAs (miRNA) delivery, peptide nucleic acids, and CRISPR-Cas technology can potentially silence any gene of interest.<sup>54–56</sup> New efforts seek to streamline evaluation of cancer genomes to allow for personalized therapies.<sup>57</sup> Improvements in sequencing technologies have evolved into new paradigms for analyzing tumor specimens; massively parallel screens can examine patient samples for potentially actionable targets.<sup>57–59</sup> Further, a pilot program, the Master Protocol, was recently approved by the Food and Drug Administration (FDA), which will connect patients to relevant drug therapy trials based on biomarkers.<sup>60</sup> Determining specific gene or biomarker expression profiles can align specific disease with the ideal, patient centered treatment regimen.

As the spectrum of genomic targets or abnormal signaling cascades widens, it may be advantageous to incorporate gene therapy along with targeted small molecules or immunotherapies. Gene delivery, however, can be either inefficient or dangerous. Similarly, most chemotherapeutics are highly toxic, especially after systemic delivery. Commonly used cytotoxic agents can act indiscriminately against both cancerous and healthy cells resulting in nausea and hair loss, neutropenia, peripheral neuropathies, kidney failure, encephalopathy, and heart disease.<sup>61</sup> The associated side effects of common chemotherapy drugs, as well as host factors and systemic delivery barriers, can severely limit dosing and, ultimately, treatment efficacy.

The complexities of cancer pathogenesis—coupled with the problems of drug resistance, side effects of therapy, and inadequate delivery to tumors—call for new solutions, especially new therapies that can overcome traditional barriers to effective treatment, while allowing for multifunctionality. Nanotechnology offers just such a solution, particularly nanoparticle drug delivery. In the next section, we discuss recent advances in the field, and promising strategies to address some of the many obstacles in cancer drug delivery.

## ■ IMPROVING CANCER THERAPIES WITH NANOTHERAPIES

**Introduction to Nanotherapies.** Nanotechnology offers the potential to improve drug solubility and stability, prolong drug half-lives in plasma, minimize off target effects, and



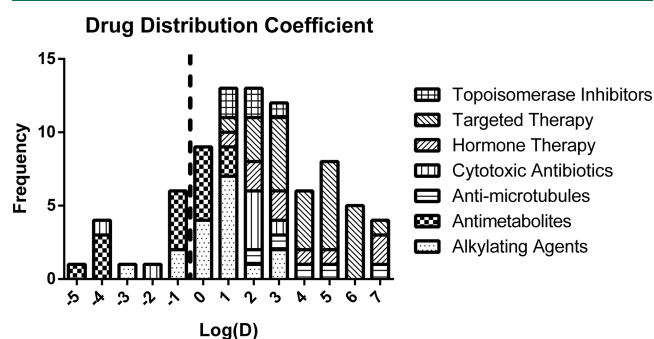
**Figure 1.** Relative sizes of nanoparticles compared to common biological structures. Illustration of nanoparticle size as compared to common biological structures and their associated length scale. An electron microscope is needed to visualize structures that are submicrometer in size.

concentrate drugs at a target site.<sup>62</sup> Nanotechnology is traditionally defined as submicron sized molecular devices or nanoparticles predominantly ranging from 5 to 500 nm in at least one dimension. Substantial past research effort has resulted in methods to incorporate therapeutic agents into biocompatible nanodevices including polymer nanoparticles, liposomes, micellar systems, inorganic nanoparticles, nanotubes, and dendrimers. Table 2 lists a few examples of nanoparticle therapeutics that are currently approved by the FDA or in clinical trials. For reference, Figure 1 compares various nanoparticles to common biological structures including hemoglobin, which is approximately 5 nm in size, to the human pupil, which is 1 000 000 times larger (4–9 mm). Nanovectors for drug delivery typically contain a core material or matrix, a therapeutic payload, and surface modifications. Of particular interest in this review are polymer nanoparticles, which have been studied extensively during the previous few decades.

**Small Molecule Delivery.** Folkman first described a method for incorporating proteins and macromolecules into polymers in 1964.<sup>75</sup> The field of polymer drug delivery has continued to evolve and generate an array of novel applications.<sup>76,77</sup> Many polymers are safe to use clinically, and the most extensively studied are poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA), which was first approved by the FDA as a suture material in 1969 and, more recently, has been approved for delivery of peptides and proteins. There are multiple PLA and PLGA delivery systems on the market including Zoladex and Nutropin Depot, and several more in the pipeline.<sup>78</sup> PLGA nanoparticles are being formulated to target specific tumors and deliver a host of agents including chemotherapy drugs or RNAi.<sup>79–81</sup> Upon exposure to physiologic solutions, PLGA undergoes hydrolysis into biocompatible metabolites, glycolic acid (GA) and lactic acid (LA), which are eventually metabolized through the citric acid cycle. Biodegradable, polymer nanoparticles provide several distinct advantages as a drug delivery vectors including tunable payload release characteristics and superior pharmacokinetics.

PLGA particles are particularly useful for agents that have low solubility in water, and therefore are difficult to formulate as drugs. The majority of clinically available chemotherapeutic

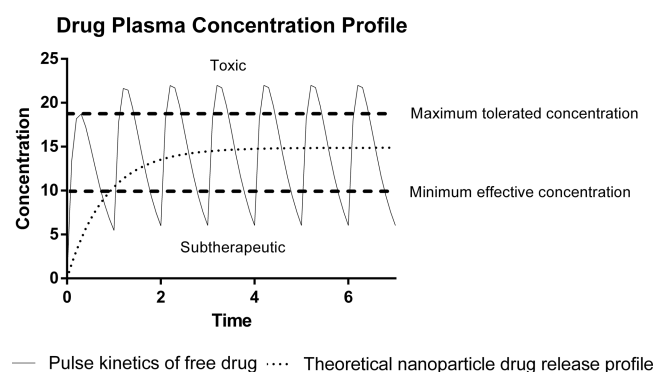
agents are lipophilic, and have low solubility in water. A common measurement of lipophilicity is the distribution coefficient,  $\log(D)$ , where  $D$  is the ratio of solute concentration in octanol to the solute concentration in aqueous buffer in both ionized and nonionized forms.  $\log(D)$  values larger than zero indicate greater solute partitioning into the hydrophobic solvent relative to water.<sup>82</sup> Figure 2 illustrates the spectrum of distribution coefficients at physiologic conditions for currently approved antineoplastic agents.



**Figure 2.** Distribution coefficient of common antineoplastic agents. The frequency distribution of antineoplastic agents by lipophilicity. The distribution coefficient ( $D$ ) is a measure of lipophilicity, and  $\log(D)$  values greater than zero indicate greater solubility in oil rather than water. The majority of clinically available antineoplastic agents are lipophilic.

The PLGA matrix releases encapsulated drugs at a sustained rate, allowing for both solubilization of drugs within the intravascular space and release over a long period. When compared to repeat free drug boluses, sustained release is more appropriate for maintaining drug concentrations within the therapeutic window. Free drug boluses result in pulsatile plasma concentrations. Levels above the minimal tolerated concentration may result in serious toxicity, and levels below the minimum effective concentration will be subtherapeutic (Figure 3). The ratio of LA to GA subunits can be adjusted to tune the rate of drug release, allowing for release profiles ranging from days to months.<sup>83</sup> Production of PLGA nanoparticles can be scaled to industrial levels, and the resulting particles can be





**Figure 3.** Nanoparticle pharmacokinetics. Drug plasma concentrations associated after repeated free drug boluses compared to a single nanoparticle dose. Because of rapid bioavailability and clearance of free drugs relative to drug encapsulated polymer nanoparticles, plasma concentrations will oscillate above and below the maximum tolerated concentration (MTC) and minimum effective concentration (MEC). Plasma drug levels above the MTC will result in systemic toxicity whereas drug levels below the MEC will be ineffective. Drug-loaded polymer nanoparticles theoretically release drugs via first-order rate kinetics resulting in a more stable plasma drug level.

stored for extended periods.<sup>84</sup> Encapsulating unstable small molecules or readily degradable proteins and oligonucleotides in a core polymeric matrix protects them from physiologic factors that would normally facilitate their clearance. Certain compounds are readily inactivated via hepatic metabolism or circulating proteases and endonucleases. Additionally, glomerular filtration in the kidneys rapidly clears compounds smaller than 10 nm. Although nanoparticles avoid renal clearance, they tend to accumulate in the mononuclear phagocyte system (MPS). But surface conjugation with polyethylene glycol (PEG) and other polymers improves particle circulation by reducing uptake into the MPS.<sup>85,86</sup> In turn, delivery via nanoparticles extends drug half-life, allowing for better control of circulating drug concentrations.

**Introduction to Gene Delivery.** Gene therapy is the cellular delivery of nucleic acids in order to modulate gene expression toward treating disease. Phenotypic modulation is achieved either through gene addition, gene correction, or gene knockdown.<sup>87</sup> Gene addition is generally the most common approach, and alters cell behavior by introducing genetic material and consequent proteins that are inherently missing in the host. Gene correction is less common, but growing in popularity, and utilizes technology—such as zinc finger nucleases, triplex forming oligonucleotides, or CRISPR-Cas—to alter or correct genomic sequences.<sup>56,88–90</sup> Finally, gene knockdown through RNAi has received significant enthusiasm. Because of the complex nature of cancer pathogenesis and multitude of signaling pathways involved in disease progression, isolating unique and singular molecular targets can become increasingly difficult. Often, tumor cells have altered transcription factor activity, influencing multiple pathways, which is difficult to target through small molecule drugs. Therefore, gene therapy can provide an alternative strategy for designing effective and specific therapies against cancer.

The U.S. FDA approved its first clinical trial in gene therapy in 1990. Michael Blease conducted an ex vivo gene therapy trial on two children with adenosine deaminase deficiency, a form of severe combined immunodeficiency (SCID).<sup>91</sup> Subsequent trials in treating SCID through ex vivo gene delivery, however, have demonstrated better long-term results.<sup>92,93</sup> In 1998, a

team in Scandinavia demonstrated the first successful gene transfer from in vivo gene delivery into the brain.<sup>94</sup> Currently, there are more than 1,800 approved clinical trials using gene therapy worldwide.<sup>95</sup> Greater than 60% of current trials are designed to treat cancer, and viral vectors continue to be the most popular approach.<sup>96</sup> China was the first country to approve a commercial gene therapy, which is currently being used to treat head and neck cancer,<sup>97</sup> and there are multiple therapies nearing the final stages of clinical testing worldwide.<sup>98</sup> Of interest, the CTL019 trial, at the University of Pennsylvania, has shown promising results using chimeric antigen receptor therapy for treating B-cell neoplasms.<sup>23,99,100</sup> The patient's T-cells are modified ex vivo using a lentiviral vector to express chimeric surface antibodies against CD19, which is expressed on B-cells. Twelve of 14 pediatric patients with acute lymphoblastic leukemia have responded to therapy, and eight experienced complete remission. Twelve of 24 adult patients with chronic lymphocytic leukemia have responded to therapy, and five of those responders have attained complete remission.<sup>101</sup>

**Methods of Gene Delivery.** There has been significant progress in the field, despite earlier setbacks, including the death of 18 year-old Jesse Gelsinger in 1999,<sup>102</sup> and the development of T cell leukemia in multiple patients receiving gene therapies for SCID.<sup>103,104</sup> The dangers of viral gene therapy are due to the associated acute immune response, immunogenicity, and oncogenesis after integration of viral components into chromosomal DNA. Even the recently successful CTL019 therapy has significant toxicities including the development of cytokine release syndrome, which can progress into macrophage activation syndrome.<sup>99</sup> Safety concerns about viral vectors, as well as their limited payload capacity and the difficulty of large-scale production, have driven interest in synthetic vectors for gene delivery. Nonviral vectors are advantageous because of their safety profile, low cost, large-scale manufacturing potential, stability, and capacity for a larger nucleic acid payload.<sup>105,106</sup> The main limitation of nonviral vectors is their low transfection efficiency. Table 3 lists the array of different vectors for gene therapy, and their associated transfection efficiency and toxicity. Although most synthetic polymers were initially considered inert, certain polymers can influence multiple cellular processes, especially when combined with biologically active agents, primarily through interactions with biological membranes and modulation of gene expression profiles.<sup>107</sup> PLGA nanoparticles can deliver nucleic acids with minimal cytotoxicity, but they have relatively low transfection efficiency. Incorporation of counterions, like spermidine, and surface functionalization with cell targeting or cell penetrating peptides have improved DNA loading and particle transfection.<sup>108,109</sup> However, their transfection efficiency remains far lower than polycationic nanoparticle formulations and viruses.

**Barriers in Gene Delivery.** Among nonviral systems, cationic liposomes are currently the gold standard. The first gene therapy trial using cationic liposomes occurred in 1992, and approximately 13% of all gene therapy trials worldwide currently use liposomal nanoparticles. Toxicity, however, is a major concern for liposomes. Additionally, liposomes are heterogeneous and relatively unstable, causing significant obstacles for large-scale pharmaceutical production.<sup>113</sup> Liposomes are readily inactivated in the serum, which can lower the high transfection levels commonly seen in vitro.<sup>114</sup> Serum instability, clearance, and cytotoxicity are common obstacles facing many nonviral gene delivery vectors. Polycationic

Table 3. Methods of Gene Delivery<sup>a,87,105,106,110–112</sup>

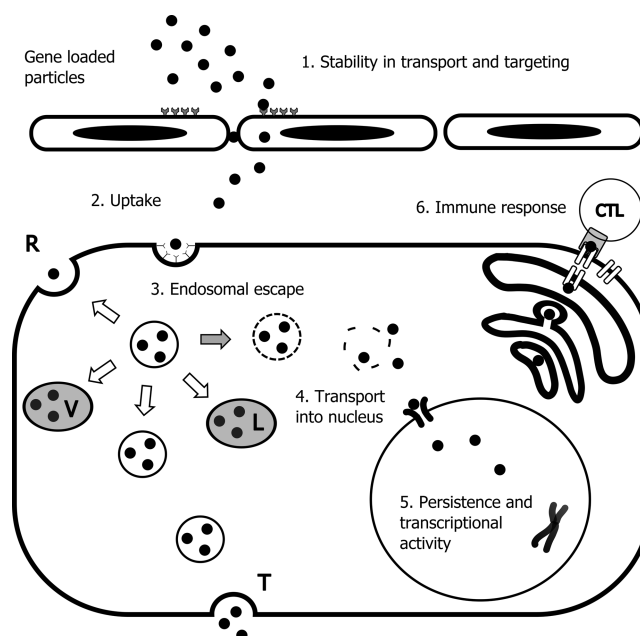
category	gene delivery system	transfection efficacy	toxicity
inorganic	calcium phosphate	II	I/II
	gold		
	magnetic		
	silica		
	quantum dots		
cationic lipids	emulsions	II/III	II/III
	liposomes		
	lipid nanoparticles		
cationic polymers	PAMAM	II/III	II/III
	PbAE		
	PEI		
	terpolymers		
cationic peptide	GALA,KALA	II/III	II/III
	poly-L-lysine		
	protamine		
	self-assembling peptides		
polymer	chitosan	II	I/II
	copolymer micelles		
	PLGA, PLA		
	polymethacrylates		
	lipid-polycationic polymer		
hybrid	PLGA-polycationic polymer	I/II	I/II
	PLGA-lipid		
	needle		
physical	ballistic DNA injection	II/III	II/III
	electroporation		
	sonoporation		
	photoporation		
	magnetofection		
	hydroporation		
viral	retroviral	III	III
	adenoviral		
	adeno-associated		

<sup>a</sup>I, low; II, medium; III, high; GALA, glutamic acid-alanine-leucine-alanine; KALA, lysine-alanine-leucine-alanine; PAMAM, polyamido-amine; PbAE, poly(beta-amino ester); PEI, polyethylenimine; PLA, polylactic acid; PLGA, poly(lactic-co-glycolic acid).

polymers, like polyethylenimine (PEI), have high in vitro transfection potential but are cytotoxic. PEI induces channel formation on the mitochondrial membrane and subsequent caspase activation and apoptosis.<sup>115</sup> Other polycations, such as poly-L-lysine (PLL) and chitosan, are less toxic but provide lower transfection. Further, PLL can stimulate an immune response due to the introduction of foreign amino acid sequences, and chitosan, at high doses can result in hypolipidemia in vivo.<sup>113</sup>

In addition to cytotoxicity, multiple barriers hinder effective transfection by nonviral systems (Figure 4).<sup>87,110,111</sup> Gene loaded particles need to protect their payload from nucleolytic enzymes while in circulation, and ultimately penetrate into the target tissue at adequate concentrations. Polymers such as PLGA encapsulate nucleic acids, protecting them from endonucleases. Similarly, condensing the negative phosphate bonds of nucleic acid chains with cationic polymers into polyplexes also protects the oligonucleotides from degradation during circulation.

After escape from the vascular space into tumors, the particle must traverse the interstitial space toward the target cell.



**Figure 4.** Barriers to gene therapy. The six major barriers for gene delivery. Gene-loaded particles need to be stable and need to protect their genetic cargo during transport in the circulatory system, while ultimately being able to localize at the target tissue.<sup>1</sup> After the tissue vasculature is penetrated, there needs to be efficient uptake of the particle into the cell.<sup>2</sup> After endocytosis, the particle needs to effectively escape the endosome<sup>3</sup> and transfer into the nucleus.<sup>4</sup> Once inside the nucleus, the transgene needs to persist and maintain adequate transcriptional activity.<sup>5</sup> During the entire process, these particles will need to evade the host immune response.<sup>6</sup> CTL, cytotoxic T lymphocyte; L, lysosome; V, vesicle; R, endosomal recycling; T, transcytosis.

Therefore, nanoparticle effectiveness depends on a variety of biophysiochemical characteristics of both the particle and cell surface. The cell surface is highly heterogeneous, both spatially and temporally, because of a variety of membrane structures, molecular interactions, and transport processes. Therefore, uptake of the vector into cells depends on both chance interaction and specific particle cell surface dynamics.<sup>116</sup> Surface charge and particle size are intimately associated with uptake efficiency. Cell surface proteins provide an overall negative charge on the plasma membrane, which readily interacts with the positive charge on certain nanocarriers. Electrostatic cell surface interactions primarily occur through positively charged vector interactions with cell surface proteoglycans. In fact, reduction in proteoglycan expression or function results in decreased transfection efficiencies.<sup>117,118</sup> Optimal particle diameters range from 50 to 120 nm, with smaller particles experiencing faster uptake.<sup>119</sup> Energy-dependent endocytosis is a primary route for cellular entry and can occur through multiple mechanisms. Recent studies report that clathrin- or caveolin-mediated endocytosis is capable of internalizing particles with diameters upward to 500 nm, yet internalization efficiency decreases with increased size.<sup>120</sup> Table 4 illustrates the five major endocytic pathways. Each route has its associated molecular players, compartment size, and intracellular fate.<sup>121–123</sup> There are multiple destinations for the early endosome, and the majority could render the genetic material ineffective. Figure 4 depicts different destinations for the endosomal cargo, and early escape appears to be necessary for subsequent gene activity. Endosomal acidification and

Table 4. Pathways for Endocytosis<sup>a,119–123</sup>

endocytic pathway	compartment size	relevant molecular players	intracellular fate	targeting modalities
phagocytosis	0.1–10 $\mu\text{m}$	actin, CDC42, PI(3), RHOA	phagosome, endosome, phagolysosome, lysosome	chitosan, mannose
macropinocytosis	50–1000 nm	ARF6, CDC42, nexins, Rab5, RAC1, rafts, ruffles	macropinosome, lysosome, TGN	AP, poly arginine peptides, TAT
clathrin coated vesicles	100–200 nm	actin, clathrins, dynamin, RHOA, SRC, TK	endosome, lysosome	antibody, RGD peptide, TAT, transferrin
noncoated vesicle (CLIC-D, CLIC-DI)	~100–500 nm	ARF6, CDC42, flotillin, RHOA	endosome, lysosome	folate, transferrin
caveolae	40–100 nm	actin, caveolin, dynamin, intersectin, lipid rafts, PKC, SRC	endosome, lysosome, golgi, ER	anticaveolae antibodies, AP, folate, TAT

<sup>a</sup>AP, antennapedia; CDC42, cell division cycle 42; CLIC-D, dynamin-dependent clathrin-independent carriers; CLIC-DI, dynamin- and clathrin-independent carriers; ER, endoplasmic reticulum; PI(3), Phosphoinositide 3; RGD, arginine-glycine-aspartic acid; TAT, transactivator of transcription; TGN, trans-Golgi network; TK, tyrosine kinases.

payload degradation through the lysosomal pathway is a common barrier for transfection. Sequestration within vesicles can also occur, as can externalization through transcytosis or endosomal recycling, all of which will reduce the effectiveness of the genetic payload. Additionally, there is significant variability in these pathways between different cell lines.<sup>124</sup>

Surface modification with ligands can facilitate particle targeting to tumors, as described above, and they can also facilitate uptake. Cell-penetrating peptides (CPPs) are short 30–35 amino acid peptides, often rich in arginine and lysine residues that promote cargo uptake via multiple mechanisms. Prototypic CPPs are the HIV transactivator of transcription (TAT) peptide and a peptide derived from antennapedia isolated from *Drosophila antennae* (AP).<sup>125</sup> Many studies have evaluated these peptides and their influence on cargo endocytosis. The specific mechanism for endocytosis varies, and several studies have suggested multiple routes of entry for individual CPPs.<sup>126–128</sup> Of interest, Zhou et al. evaluated histidine modifications of several CPPs, and found that flanking AP with five histidine residues (mAP) significantly increased nanoparticle transfection efficiencies.<sup>108</sup> Additionally, particle surface modifications with antireceptor antibodies, or surface conjugation with folate and transferrin can increase cargo uptake.<sup>129–132</sup> Table 4 summarizes targeting strategies for specific endocytic pathways.

Strategies to improve endosomal escape include utilizing fusogenic or pore-forming peptides or the proton sponge effect.<sup>132</sup> Certain viral peptides are known to promote endosomal disruption in a pH-dependent manner, and similar peptides have been synthesized to improve gene transfection when incorporated with DNA polyplexes.<sup>133</sup> These polymers are converted from hydrophilic to hydrophobic structures upon protonation in the endosome, which consequently allows for vesicle membrane lysis.<sup>134</sup> Cationic polyplexes, such as PEI or polyamidoamine (PAMAM) dendrimers, can also promote endosomal escape through the proton sponge mechanism.<sup>135</sup> During endosomal acidification, the protons pumped into the lumen via the vacuolar-ATPase are buffered by amines on the polymers. This buffering leads to an increase proton influx, which passively recruits chloride ions in order to maintain charge balance. The resulting accumulation of osmotic agents results in endosomal swelling and consequent lysis. Additionally, codelivery of chloroquine analogues has been shown to improve gene transfection through unclear mechanisms. There is some evidence that chloroquine can either act as a pH buffer, displace cationic complexes from nucleic acids, or alter biophysical properties of the released genetic material.<sup>136</sup>

If the nanocarrier does escape the endosome, depending on the genetic payload, the next challenge is nuclear targeting. Plasmid delivery requires translocation into the nucleus to attain transcription, whereas siRNA or miRNA activity resides in the cytoplasm. Additionally, cytosolic nucleases will eventually degrade cytosolic DNA or RNA, so effective translocation must occur prior to degradation. A variety of approaches appear to stabilize nucleic acids during transport. Polyplexes can potentially protect against cytosolic nucleases and travel along microtubules toward the nucleus via nonspecific charge interactions or even motor-protein driven transport.<sup>137</sup> Modulation of microtubules via histone deacetylase inhibitors has improved transfection by 10-fold.<sup>138</sup> Additionally, random redistribution during mitosis can result in gene uptake within the nucleus.<sup>139</sup> Nuclear localization signals (NLS), which are naturally occurring cationic peptides, are used to deliver proteins to the nucleus. Polyplexes may act similarly to NLS because of their inherent positive charge. NLS can also be conjugated to plasmids to improve nuclear targeting and transfection.<sup>140</sup> Co-delivery of *trans*-cyclohexane-1,2-diol (TCHD) has been shown to improve gene transfection through nonselective gating of the nuclear pore.<sup>141</sup> Within the nucleus, vector genome persistence may be an issue if the exogenous material does not integrate with the host genome. The episomal DNA can persist in quiescent tissue; however, gene expression will become increasingly transient in rapidly dividing cells. Repeat dosing may then be required to sustain therapeutic transfection levels. Vector integration into the host genome can lead to greater persistence at the risk of gene disruption via insertional mutagenesis. Additionally, epigenetic alterations may disrupt gene expression regardless of genome integration. Persistence and sustained gene expression are vital for diseases requiring permanent gene expression. For acquired diseases like cancer, transient transfection may be adequate to achieve a therapeutic effect. Alternatively, minicircle DNA vectors lacking bacterial DNA have been expressed at high levels in vivo for extended periods, yet obstacles in their mass production have limited their use.<sup>142</sup> There has been significant strides to improve minicircle production using carefully engineered plasmids and culturing techniques.<sup>87</sup>

The host immune response is a significant barrier to efficient gene therapy. Particle components, extranuclear nucleic acids, and transgene products can activate an immune response. It is difficult to predict human immune responses because most animal models fail to replicate the human immune systems accurately. Generally, the immune response elicited by viral vectors is far more severe than that of nanoparticles, as most



particles are only as immunotoxic as their cargo. However, foreign DNA payloads can also illicit an interferon response that can potentially lead to immunotoxicity or decreased transfection efficiencies.<sup>143</sup> The inflammatory response may be due to the presence of unmethylated CpG dinucleotides present on plasmid DNA. Mutating the immunostimulatory CpG motifs or codelivering immunosuppressants can decrease the inflammatory response and elevate transgene expression.<sup>144,145</sup> Additionally, higher surface charges present on liposomes have induced secretion of cytokines including tumor necrosis factor (TNF), interleukin 12, nuclear factor  $\kappa\beta$  (NF $\kappa\beta$ ), and interferon  $\gamma$ . However, these immunostimulatory effects may be beneficial in cancer therapies.<sup>146–148</sup>

The MPS plays a major role in nanoparticle clearance. Nanoparticles readily adsorb plasma proteins upon introduction to systemic circulation, and are consequently opsonized and phagocytized by the MPS. Due to hepatic and splenic filtration, particles tend to accumulate in those organs.<sup>149</sup> Smaller particles and a neutral surface charge result in lower levels of opsonization and phagocytosis. Surface modifications can reduce protein adsorption and entrapment within the MPS. For instance, PEG not only decreases protein adsorption and phagocytis into the MPS as discussed earlier, but it can also decrease platelet and erythrocyte interactions.<sup>150</sup> Unfortunately, the shielding effects of particle PEGylation are transient and can hinder target particle cell interactions as well. Poloxamer and poloxamine have been evaluated as potential alternatives to PEG, and have been shown to have similar benefits and drawbacks.<sup>151,152</sup> Additionally, surface functionalization with self-peptides, like CD47, can delay macrophage-mediated clearance.<sup>153</sup> Nanoparticle design is critically important to address the multiple barriers involved in gene transfection; Table 5 summarizes the common strategies utilized in particle engineering.

**Targeting Tumors with Nanoparticles.** Nanoparticle systems also have unique properties that allow for both passive and active targeting of tumors. Because of up regulation of proangiogenic signaling, most solid tumors are hypervascular. However, the new vessels have abnormal architecture and are highly permeable. The tumor mass also has poor lymphatic drainage, allowing for accumulation of macromolecules greater than approximately 40 kDa within its microenvironment. Nanoparticles exploit this feature, which is called the enhanced permeability and retention (EPR) effect, to target solid tumors. The ideal size range to benefit from the EPR effect is between 10 to 200 nm. Particles that are too small will be renally cleared, preventing accumulation into the tumor site, and particles that are too large will not adequately penetrate the tumor vasculature and interstitial space.<sup>154,155</sup>

Particle surface modifications can be incorporated to improve cell targeting and internalization while bypassing certain forms of multidrug resistance.<sup>156</sup> Nanoparticles coupled with surface ligands or antibodies can localize to tissue expressing the associated receptors or antigens and improve delivery efficacy.<sup>157</sup> Certain ligand receptor interactions will facilitate receptor-mediated endocytosis, which can further enhance payload delivery. Surface ligand or antibody coupling can achieve densities high enough to interact efficiently with target sites, and these techniques lend themselves well to cancer therapies.<sup>158</sup> New antibody-coated nanoparticles have even allowed for effective oral delivery.<sup>159</sup> Many tumors up-regulate growth factor receptors, such as ErbB2 in certain breast cancers, which can be targeted with anti-ErbB2 surface antibodies.<sup>157</sup>

**Table 5. Strategies for Addressing Gene Delivery Barriers via Nanoparticles<sup>a</sup>**

barrier	strategy
1. stability in transport and targeting	local delivery <sup>156</sup> encapsulation in lipid and polymer delivery systems tumor homing peptides <sup>161</sup> fabricate at optimal particle size for the EPR effect <sup>155,164</sup>
2. uptake	fabricate at optimal particle size for cellular uptake <sup>119</sup> ligand or CPP surface modifications <sup>63,126–128,131,156,165</sup>
3. endosomal escape	chloroquine analogues <sup>136</sup> pH sensitive, fusogenic, or synthetic peptides <sup>152,133</sup> histidine-rich peptides <sup>132</sup> “proton sponge” polymers <sup>135</sup>
4. transport into nucleus	nuclear pore gating with TCHD <sup>141</sup> nuclear targeting via NLS <sup>124,132,140</sup> transport along microtubules <sup>137</sup> HDAC inhibitors <sup>138</sup>
5. persistence and transcriptional activity	insertional vectors <sup>87</sup> minicircle DNA vectors <sup>87,142</sup> repeat dosing <sup>87</sup>
6. immune response	co-deliver immunosuppressants <sup>145</sup> fabricate at optimal particle size and surface charge <sup>149</sup> mutating immunostimulatory CpG motifs on plasmid DNA <sup>144</sup> surface modifications with PEG, poloxamer, or poloxamine <sup>150,151</sup> surface functionalization with self-peptides <sup>153</sup>

<sup>a</sup>EPR, enhanced permeation and retention; HDAC, histone deacetylase; PEG, polyethylene glycol; NLS, nuclear localization signal; TCHD, trans-cyclohexane-1,2-diol.

Various cancer lines up regulate surface antigens, including fetoprotein, human carcinoembryonic antigen, and human chorionic gonadotropin antigen, which provide targets for antibody mediated targeting.<sup>63</sup> Additionally, CPPs or targeting peptides can facilitate interactions with tumor cells or tumor endothelium. Established conjugation chemistries provide facile mechanisms for surface modifying polymer nanoparticles with targeting peptides. Zhou et al.<sup>108</sup> optimized multiple CPPs for improved particle endocytosis and Teesalu et al.<sup>112,160</sup> used the novel internalizing RGD (iRGD) peptide to target nanoparticles to the tumor endothelium. The RGD peptide sequence recognizes the  $\alpha_v\beta_3/\alpha_v\beta_5$  integrins that are up-regulated on tumor endothelial cells. The cyclic iRGD peptide contains both the RGD sequence and a CendK/R element. The iRGD structure is readily cleaved by proteases, allowing for exposure of the CendK/R element and subsequent tissue penetration through binding of neuropilin-1.<sup>161</sup>

Active nanoparticle targeting as well as particle clearance by the liver, lungs, and MPS can be beneficial for treating advanced cancers and metastatic disease, which has proven to be resistant to conventional methods.<sup>162</sup> In addition to ligand-based targeting, nanoparticles can be delivered locally via intravenous catheters, inhalation, transdermal patches, or intravitreal administration.<sup>156,163</sup> Local delivery of chemotherapeutic agents can minimize several of the harmful side effects associated with common cancer therapies. New polymer delivery vehicles allow for targeted and combination cancer therapies, which can



ultimately decrease the development of drug resistance while simultaneously minimizing the side effect profile.

**Combination Therapies.** Delivering multiple agents in vivo is complicated because of their independent pharmacokinetics, biodistribution, and clearance.<sup>166</sup> Nanoparticle delivery systems can consolidate these properties into one vehicle and increase the likelihood that targeted tumor cells receive both agents at a ratiometric dose.<sup>167</sup> Therefore, once an optimal drug ratio is tuned in vitro, it can be translated to the clinic effectively. Combining multiple agents into one carrier can also streamline manufacturing and infusion processes, overcome batch-to-batch variability, and lower costs. The patient will also receive smaller doses of the nanocarriers, which can potentially lower toxicity. There have been several reports of codelivering multiple anticancer agents using nanocarriers, and some are reaching clinical trials.<sup>168–172</sup> Only a few of these approaches are capable of efficiently codelivering both small molecule drugs and genetic material in vivo.<sup>170,173–175</sup> As these carriers show considerable promise, improvements are still necessary to improve transfection potential, while maintaining ideal particle size, surface charge, loading, targeting, and biocompatibility. Additionally, evaluation of synergistic interactions is rare, especially when using nanoparticle vectors for codelivery, and several of the reported nanocarriers were only able to show improved anticancer effects at high doses. Table 6 highlights interesting nanoparticle formulations that effectively analyzed the delivery system for synergy, and were translated effectively to in vivo antitumor therapies.

**Table 6. Combination Nanoparticle Formulations Translated to Effective in Vivo Antitumor Therapies<sup>a</sup>**

formulation	therapeutics	synergy analysis
CPX-351 liposome (phase II clinical trials) <sup>176</sup>	cytarabine and daunorubicin	yes
CPX-1 liposome (phase II clinical trials) <sup>177</sup>	irinotecan and floxuridine	yes
CPX-571 liposome <sup>178</sup>	irinotecan and cisplatin	yes
pegylated liposome <sup>179</sup>	quercetin and vincristine	yes
triblock polymer micelle <sup>180</sup>	paclitaxel and Plk-1 siRNA	yes
PEGylated dendrimers <sup>173,174</sup>	doxorubicin and TRAIL encoded plasmid	yes
PLGA core with surface PEI and PEG <sup>181</sup>	camptothecin and TRAIL encoded plasmid	yes
PLGA core with block copolymer envelope <sup>168</sup>	doxorubicin and combretastatin	no
cationic amphiphilic copolymer <sup>170</sup>	paclitaxel and IL-12 encoded plasmid	no
pegylated liposome <sup>175</sup>	doxorubicin and c-Myc siRNA	no
aptamer-dendrimer conjugates <sup>182</sup>	doxorubicin and immune stimulating unmethylated CpG oligonucleotides.	no
dendritic PEG <sup>183</sup>	paclitaxel and alendronate	no

<sup>a</sup>IL-12, interleukin-12; PEG, polyethylene glycol; PEI, polyethylenimine; PLGA, poly(lactic-co-glycolic acid); Plk-1, polo-like kinase 1; siRNA, short interfering RNA.

Designing therapies with synergistic agents allows for reduced drug dosing and toxicity. Two agents act synergistically when their combined effect is greater than the sum of their individual effects. Analysis of synergism is complex and there are numerous methods for determining true synergism. Several of these methods contradict each other. Although commonly presented, the arithmetic sum of individual effects does not

necessarily provide a cutoff for synergism, since you cannot have effect levels greater than one. Potency as well as efficacy is important in determining synergy, and therefore a dose response curve is necessary for accurate analysis. Chou and Talalay derived the combination index (CI) and median effect equation (MEE) in 1984, and have since established precedents for analyzing synergism.<sup>184,185</sup> The MEE is derived from the mass action law, and describes the behavior of many biological systems. In fact, the Michaelis–Menten, Hill, Henderson–Hasselbalch, and Scatchard equations can be derived from the MEE. Therefore, the mechanisms of action and conventional kinetic constants for the individual agents are not necessary to evaluate synergism via this method.

The linearized MEE uses dose response data to determine  $D_m$ , the median effective dose, and  $m$ , a Hill-type coefficient (eq 1). The coefficient for the linear regression is an indicator for the applicability of the Chou–Talalay analysis. At each effect level, the corresponding doses,  $D_{\text{alone},1}$ ,  $D_{\text{alone},2}$ ,  $D_{\text{comb},1}$ , and  $D_{\text{comb},2}$ , can be tabulated to determine a CI value using eq 2. CI values less than one indicate synergistic effects, values greater than one indicate antagonistic effects, and values equal to one indicate additive effects.<sup>184,185</sup> The doses for individual agents during the combined dose response analysis are determined using the known ratio between the two components. Finally, a calculated dose-reduction index (DRI) can provide the fold change in drug dosing required to achieve a similar effect (eq 3). A computer program, CompuSyn, is available to assist with the Chou–Talalay analysis.<sup>186</sup>

$$\log\left(\frac{f_a}{f_u}\right) = m\log(D) - m\log(D_m)$$

$$f_a + f_u = 1$$

$$\log(f_u^{-1} - 1) = m\log(D) - m\log(D_m) \quad (1)$$

$$CI = \frac{D_{\text{comb},1}}{D_{\text{alone},1}} + \frac{D_{\text{comb},2}}{D_{\text{alone},2}} + \alpha \frac{D_{\text{comb},1}D_{\text{comb},2}}{D_{\text{alone},1}D_{\text{alone},2}} \quad (2)$$

Where CI is combination index;  $D_{\text{alone},1}$ , dose of drug 1;  $D_{\text{alone},2}$ , dose of drug 2;  $D_{\text{comb},1}$ , combination dose of drug 1;  $D_{\text{comb},2}$ , combination dose of drug 2. For mutually exclusive drugs,  $\alpha = 0$ , and for mutually nonexclusive drugs,  $\alpha = 1$ .

$$DRI_1 = D_{\text{alone},1}/D_{\text{comb},1} \quad (3)$$

Where DRI is dose-reduction index;  $D_{\text{alone},1}$ , dose of drug 1; and  $D_{\text{comb},1}$ , combination dose of drug 1.

## ■ CHALLENGES AND FUTURE DIRECTIONS

The EPR effect is often cited to support nanoparticle delivery into tumors. Yet, there is considerable variability of the EPR effect between not only the vasculature within tumors but also between tumor types and tumor models.<sup>187</sup> Therefore, it is difficult to predict clinical efficacy based on preliminary in vivo data. In fact, only 8% of successful animal studies are translated into clinical trials.<sup>188</sup> In clinical practice, it may be necessary to evaluate EPR activity in each specific patient with diagnostic nanoparticles. It is also possible to enhance the EPR effect by utilizing active tumor targeting, or coadministering agents to augment tumor vasculature and blood pressures.<sup>189</sup> Multiple studies evaluate nanoparticle efficacy using subcutaneous tumor

models, which may falsely overestimate high EPR vasculature. Although, there may be some limitations with utilizing the EPR effect, the technology to actively target to tumors can potentially overcome those shortcomings.<sup>187</sup> Metastatic, orthotopic, and genetically engineered animal models should provide more accurate cancer models, but need additional characterization. Advanced tumors will cause greater animal morbidity requiring earlier termination. It may be beneficial to utilize a hierarchical approach, where researchers screen with small subcutaneous xenografts and continue to evaluate therapies against larger tumors, orthotopic grafts, and ultimately with genetically engineered cancer models.<sup>190</sup>

Additional limitations for nanotherapy in cancer continue to be particle clearance and, in the case of gene delivery, inefficient transfection. This is complicated by the observation that particle properties that promote higher cellular uptake or gene transfection usually also produce greater cytotoxicity or clearance in vivo. In the case of combination therapies, careful synergy analysis is needed to provide the optimal ratio of therapeutics and potentially a lower loading requirement for the delivery vector. The field of cancer nanotherapy continues to make significant advancements through both increasing vector functionality and tumor targeting. There are many barriers to drug and gene delivery, yet the multifunctionality of nanoparticle systems allows for rational, stepwise optimizations for addressing these barriers. The evolving sophistication of nanotherapy systems allows for addressing heterogeneity and biological diversity that are now known to exist within the most aggressive cancers. For instance, new nanoparticle techniques are being used to target cancer stem cells which may be linked to treatment resistance.<sup>191,192</sup> And given their unique interactions with the immune system, nanoparticles are being evaluated as therapeutic vaccines, targeted monoclonal antibody treatments, and activators of cell based immune therapies.<sup>193</sup> The ability to deliver multimodal therapeutic agents can potentially provide synergistic therapies, leading to decreased dosing and associated toxicities. Improvements in materials design are providing new functionalities for therapeutic encapsulation and surface ligand conjugations for tumor targeting. Nanoparticle design has become increasingly sophisticated over the past few decades, and a rational approach to their design can potentially address many of the obstacles in current cancer therapies.

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### Notes

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