

Advances in Drug Delivery

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Abstract

In this article, we review critical aspects in the area of drug delivery. Specifically, delivery of siRNA, remote-controlled delivery, noninvasive delivery, and nanotechnology in drug delivery are reviewed.

INTRODUCTION

The delivery of drugs and biological agents is an area of immense importance for human health. Although barely existing some 30 years ago, such systems are now being utilized by tens of millions of patients every year. Such drug delivery systems have prolonged life and relieved suffering and may eventually enable the clinical use of new biological agents [e.g., short interfering RNAs (siRNAs)]. Here, we discuss four important areas in this field: (a) the delivery of siRNAs, (b) the development of remotely triggered delivery systems, (c) different routes of delivery through the body, and (d) targeted nanoparticles.

DELIVERY OF SHORT INTERFERING RNAS

Since the discovery of RNA interference (RNAi) and siRNA a decade ago (1, 2), RNAi therapeutics have demonstrated considerable potential in the treatment of a wide variety of human diseases, including viral infections (3, 4), hereditary disorders (5), and cancer (6, 7). Theoretically, siRNA-based therapeutics can modify the expression of nearly any existing gene in the body, giving them a broader therapeutic potential than that of conventional small-molecule drugs. Synthetic siRNAs have reportedly treated a wide variety of disease targets in vivo, including hypercholesterolemia (8), liver cirrhosis (9), Huntington disease (5), hepatitis B virus (3), human papillomavirus (10), and melanoma (11).

RNAi is a fundamental pathway conserved in eukaryotic cells that mediates silencing of genes at the mRNA level as well as silencing of any encoded proteins (12). When siRNA is introduced into the cytoplasm of a cell, it is bound by the RNA-induced silencing complex (RISC). RISC unwinds the siRNA duplex, discards the sense strand, and escorts the antisense strand to a complementary mRNA sequence, which it then cleaves (13, 14). Upon siRNA delivery, silencing of mRNA can persist anywhere from several days to several weeks, depending on the siRNA dose and the rate of target cell turnover.

For siRNA therapeutics to be implemented in a clinical setting, safe and effective delivery systems must be developed to guide the siRNA into the cytoplasm of the target cell. Although “naked” siRNA has shown efficacy in certain clinical settings (5, 15), the majority of organ targets in the body require a delivery vehicle to facilitate transfection. This is because naked siRNA is subject to various clearance mechanisms in vivo and is too large (~13 kDa) and too negatively charged to cross cellular membranes. Efficacious and nontoxic delivery is a key challenge and poses the most significant barrier between siRNA biotechnology and its therapeutic application.

In Vivo Barriers to Systemic Short Interfering RNA Delivery

Most RNAi therapeutics necessitate the systemic administration of siRNA delivery complexes through the bloodstream. Nanoparticle formulations for systemic injection must navigate a series of in vivo obstacles before engaging the RNAi machinery in the cytoplasm of the target cell. Postinjection, the siRNA complex must avoid kidney filtration, uptake by phagocytes, aggregation with serum proteins, and enzymatic degradation by endogenous nucleases (16). Transport out of the bloodstream and across the vascular endothelial barrier poses a significant challenge for delivery to many tissues within the body. Although particles larger than 5 nm do not typically cross endothelial membranes, certain tissues, including liver, spleen, and some tumors, permit the entry of drug delivery particles of up to 200 nm in diameter (17). Once an siRNA delivery complex diffuses through the extracellular matrix, it must be uptaken into the target cell, typically through an endocytic mechanism. Particles must then escape the endosome to reach the cytoplasm before endosomal/lysosomal degradation occurs (18, 19). Finally, siRNA must be released to the cellular machinery if the siRNA was formulated with delivery agents.

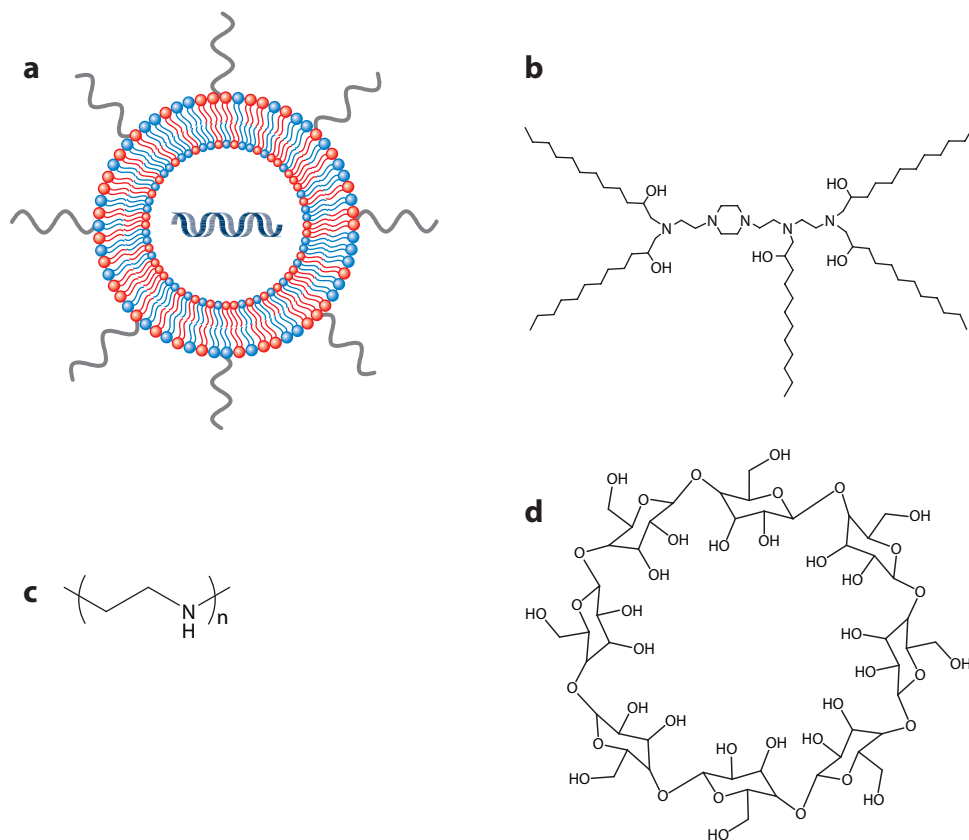


Figure 1

Synthetic cationic materials for siRNA delivery. (a) A representation of stable nucleic acid-lipid particles (SNALPs). The liposomes are formulated with cationic lipids (*red*), nonionic lipids (*blue*), and poly(ethylene glycol) (*gray*). siRNA is contained in the hydrophilic interior of the particle. (b) The lipidoid C12-200. (c) A polyethyleneimine monomer can be used to generate both linear and branched polymeric delivery systems. (d) A cyclodextrin monomer.

Cationic Materials for Short Interfering RNA Delivery

Synthetic cationic materials have demonstrated considerable potential as nonviral siRNA delivery vehicles (**Figure 1**). Cationic materials offer several benefits, including the ability to facilitate cellular uptake through contact with the negatively charged cellular membrane (20). Through electrostatic interactions, a positively charged delivery vehicle enables complex formation and compression with negatively charged siRNA. Additionally, cationic materials become more protonated as pH drops, which can potentially assist in proton sponge-mediated endosomal escape (18). This subsection focuses on synthetic cationic materials that have demonstrated an ability to potently silence therapeutic targets, with a focus on the more recent literature.

Liposomes and lipid-like materials. Nucleic acids have been delivered via liposomes for more than 20 years, originating with Felgner and colleagues' (21, 22) studies describing the use of DOTMA, a cationic lipid, to deliver both DNA and RNA into multiple cell lines. Currently, one of the most successful liposomal delivery techniques involves the stable nucleic acid-lipid particle

(SNALP), which has demonstrated efficacy in multiple in vivo models. The first group of work involves SNALP delivery to the liver. In 2005, siRNA-SNALP complexes were shown to inhibit hepatitis B virus (HBV) replication through the delivery of an anti-HBV siRNA. Results were dose dependent, with three siRNA doses of $3 \text{ mg kg}^{-1} \text{ day}^{-1}$ able to reduce serum HBV levels for up to 7 days after dosing (3). In the first report of successful siRNA delivery in nonhuman primates, Zimmerman and colleagues (23) used SNALP to silence apolipoprotein B (ApoB) in the liver of cynomolgus monkeys. In 2009, SNALPs encapsulating two essential cell-cycle proteins displayed potent antitumor efficacy in both hepatic and subcutaneous tumor models (24). Most recently, one of the key lipid components of SNALP, DLinDMA, was modified and optimized in the context of SNALP vehicles to enable endogenous hepatic gene silencing at siRNA doses as low as 0.01 mg kg^{-1} (25).

Several studies from McLachlan's group (4, 26) have detailed the use of SNALP nanoparticle formulations to treat the Zaire Ebola virus, which is excruciating and highly fatal in humans. An initial report describes the use of siRNA targeting the polymerase (L) gene of the virus to completely protect guinea pigs against viral infection and death when delivered following an Ebola virus challenge (26). A subsequent study demonstrated that seven 2 mg kg^{-1} postexposure doses of nonimmunostimulatory siRNA-SNALP complexes protected all three rhesus macaques studied (4).

Lipidoids are another class of lipid-like materials that has been developed for use in siRNA delivery applications. These materials are synthesized through the conjugate addition of alkyl-acrylates, alkyl-acrylamides, and alkyl-epoxides to primary and secondary amine molecules. The original study on lipidoids described the use of 98N₁₂-5(1), which comprises five 12-carbon alkyl-acrylamide chains attached to an amine core (27). When formulated with siRNA, 98N₁₂-5(1) potently and persistently silenced various lung and liver targets in mice, rats, and cynomolgus monkeys. In another primate study, 98N₁₂-5(1) was used to silence the hepatic low-density lipoprotein receptor PCSK9, demonstrating an approach that may serve as an effective treatment for hypercholesterolemia (8). *Hmox1*, a liver gene encoding the protein heme oxygenase-1, has also been silenced in mice by siRNA nanoparticles formulated with lipidoids, which may have applications in malarial infection and disease (28). Most recently, Love and colleagues (29) have identified a new lipidoid delivery molecule, C12-200, which is the most efficacious lipidoid to date. C12-200 mediates liver gene silencing in nonhuman primates at doses as low as 0.03 mg kg^{-1} and enables the simultaneous silencing of five hepatic gene targets with a single injection (29).

Polymers. Polyethyleneimine (PEI) is a broadly investigated delivery carrier for the administration of a wide array of nucleotide-based therapies, including DNA, siRNA, and oligonucleotides (30, 31). Multimerized siRNA that were cross-linked through cleavable disulfide linkages induced gene silencing in mouse tumor xenografts when the siRNA was delivered with linear PEI (32). Multimerized siRNA formed more stable complexes with PEI than did native 21-base-pair siRNA, which may explain why the multimerized siRNA facilitated higher levels of gene silencing both in vitro and in vivo.

Polymer-siRNA conjugates have also shown potential for applications in systemic siRNA delivery. Rozema and coworkers (33) have developed a conjugated polymer delivery system named Dynamic PolyConjugates that can mediate siRNA delivery to hepatocytes. These cationic polymers were able to induce the silencing of two mouse liver genes, including those encoding ApoB and peroxisome proliferator-activated receptor α , without inducing any apparent toxicity (33).

Poly(β -amino ester) (P β AE) nanoparticles, although traditionally used in DNA delivery applications (34, 35), have also enjoyed some success in the RNAi therapeutics arena. In 2008,

Vandenbroucke and colleagues (36) employed P β AEs to successfully provoke gene silencing in primary mouse hepatocyte cell culture. A separate study demonstrated that, through electrostatic interactions, P β AEs can be used to coat siRNA-conjugated gold nanoparticles (37). These coated nanoparticles can then be delivered into cell culture to mediate dose-dependent gene knockdown.

Cyclodextrin polymers have also been developed as siRNA delivery reagents. In 2007, targeted transferring cyclodextrin nanoparticles were used to study the effects of siRNA delivery on the immune system of nonhuman primates. This study reported that repeated doses of targeted nanoparticles containing nonchemically modified siRNA can be safely administered systemically to cynomolgus monkeys (38). Most recently, as part of a phase I clinical trial, evidence of an RNAi mechanism was reported in human tumor tissue after delivery of a targeted cyclodextrin-siRNA nanoparticle (11).

Future Directions for Short Interfering RNA Delivery

The field of RNAi and siRNA therapeutics has made significant strides in the past decade toward clinical applications. In the future, synthetic cationic materials, including lipid-based molecules and polymers, should continue to play an important role in the safe and efficacious delivery of siRNA. Future developments will require an attention to the immunostimulatory potential of siRNA and the modification methods that can be used to engineer siRNA that does not activate the innate immune system (39–43). Delivery continues to pose the most significant barrier to the clinical use of RNAi therapeutics, and future work centering on the development of effective, nontoxic delivery vehicles will need to be carried out to facilitate the broadest clinical application of RNAi.

REMOTELY TRIGGERED DELIVERY SYSTEMS

Systems that release drugs in response to a remote trigger, that is, systems that are actuated by an external stimulus, could offer distinct clinical advantages over those that release drugs passively or are triggered internally, e.g., by a chemical stimulus. Drug release profiles could be tailored to the specific therapy. For example, insulin is most effective when delivered to a diabetic in short bursts (44), whereas an anesthetic should be delivered in a steady, continuous fashion (45). Most importantly, perhaps, remotely triggered systems would allow the patient or doctor to control the dose, timing, and duration of drug release.

Numerous stimuli could be used for drug triggering, including visible or near-infrared (NIR) light, electric or magnetic fields, and ultrasound (46). Some of these techniques, such as ultrasound, magnetic resonance imaging (MRI), and NIR fluorescence microscopy, are already used in the clinic or lab for imaging. Recently, investigators have developed functional materials that are sensitive to these stimuli and that have been used to design drug delivery systems.

Near-Infrared Light

NIR (~650–900-nm) light is a promising tool for drug delivery because it can penetrate skin and tissue, enabling triggering deep within the body (47). Investigators have developed a variety of nanostructures such as gold nanorods, nanoshells, and nanocages that absorb strongly in this range and that convert light energy into heat (48). Hence, gold nanoparticles can behave as localized heat sources, and they have been used to trigger drug release from temperature-sensitive materials, as shown schematically in **Figure 2**.

One temperature-sensitive material that has been used extensively is poly(*N*-isopropylacrylamide) (pNIPAm), a polymer that abruptly and reversibly collapses when

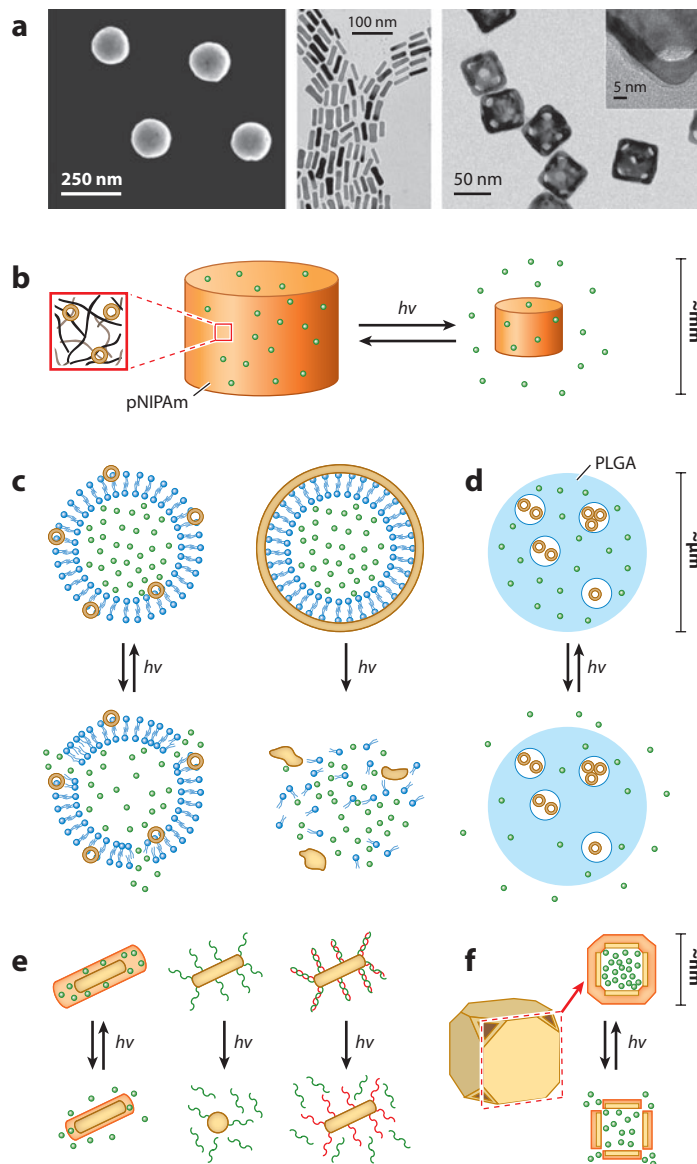


Figure 2

Triggered drug release using near-infrared (NIR)-active gold nanoparticles. (a) Examples of gold nanoparticles: (left) shells, (center) rods, and (right) cubes. The nanoparticles function as local heat sources when irradiated with NIR light ($h\nu$). The micrographs shown are, from left to right, from References 49, 53, and 59, with permission. (b) Macroscale poly(*N*-isopropylacrylamide) (pNIPAm)-gold nanoshell composites collapse when heated beyond a critical temperature, expelling drug (green spheres). (c) Temperature-sensitive liposomes are (left) disrupted or (right) destroyed by local heating effects and release encapsulated drug. (d) A hydrophobic drug confined in poly(lactic-*co*-glycolic acid) (PLGA) is released when heated by gold nanoshells. (e) Drugs or oligonucleotides can be released from nanorods. (Left) Rods are coated with pNIPAm that collapses upon irradiation. (Center) Oligonucleotides are tethered to the surface of the gold and are released when the particle is deformed. (Right) Double-stranded DNA melts when heated by the nanorod. (f) Hollow gold nanocubes exhibit pores that are sealed by pNIPAm; these pores are exposed when the polymer collapses, enabling drug efflux.

heated beyond a critical temperature. If the polymer is loaded with drug in its swollen state, the drug will be expelled upon collapse. Initial studies on NIR triggering dealt with gold nanoshells (49) incorporated into bulk pNIPAm (or pNIPAm derivatives exhibiting different critical temperatures), which collapsed and released small or macromolecules upon NIR irradiation (50, 51). This technique was extended to gold nanorod/pNIPAm core/shell particles, which exhibited short, pulsatile drug release upon collapse of the polymer shell (52). pNIPAm has also been used to coat gold nanocages, which exhibit hollow centers and porous walls. In the swollen state, pNIPAm blocked the pores and inhibited drug efflux. When collapsed, the pores opened, and the drug freely diffused out of the structure (53).

Liposomes have also been used extensively as drug carriers, and temperature-sensitive varieties have been functionalized with gold nanoshells (54) or a dense coating of plasmonically coupled gold nanospheres on the outer surface of the liposome (55). In both cases, local heating induced by NIR irradiation rendered the surface of the liposomes porous and released the encapsulated drug. Drug-containing liposomes have also been encapsulated with solid gold shells. These structures exhibit the benefit of minimal baseline leakage because of the solid shell, whereas irradiation releases drug by deforming the shell and breaking the liposome (56).

Triggered release of the hydrophobic drug paclitaxel (PTX) was recently demonstrated from biodegradable, biocompatible poly(lactic-co-glycolic acid) (PLGA) microspheres, into which gold nanoshells had been incorporated. Under NIR irradiation, localized heating by the nanoshells drove PTX out of the microspheres, with release kinetics dependent upon irradiation intensity. These particles were injectable and demonstrated antitumor effects on human gliomas grown in rat models (57).

Molecules can also be released directly from the surface of a gold nanoparticle. For example, femtosecond NIR laser pulses have been used to melt nanorods into nanospheres, releasing surface-bound ligands such as DNA as the gold atoms rearrange (58, 59). Laser pulses have also been used to release DNA oligonucleotide ligands by heating and dehybridizing the helix, but not melting the nanoparticle (60). Functionalized nanorods are readily taken up by living cells, and both of these techniques have been used in vitro to modulate gene expression.

Magnetic Fields

Drug elution triggered by magnetic fields was first demonstrated by embedding millimeter-scale magnetic bars in poly(vinyl alcohol) (PVA); baseline release rates of drugs increased five- to tenfold under application of an alternating magnetic field, a result of physical deformation of the polymer by spinning rods (44).

Recent studies have utilized superparamagnetic nanoparticles such as iron oxide crystals of the ferrite (Fe_3O_4) or maghemite ($\gamma\text{-Fe}_2\text{O}_3$) structures, which exhibit single magnetic domains and release heat when exposed to alternating magnetic fields. Hence, these structures are similar to gold nanoparticles in that they can be used as local heat sources in temperature-triggered systems. They have been used to trigger drug release by incorporation into a variety of systems: macroscale polymers [e.g., pNIPAm (61) or chitosan (62)], microspheres (63, 64), liposomes (65), micro- or nanocapsules (66–68), and nanospheres (69–71).

Engineered stimulus-sensitive membranes can be used as the walls of reservoirs, enabling a large quantity of drug to be contained and released repeatedly over extended periods. Nanocomposite membranes consisting of pNIPAm-based nanogels and magnetic nanoparticles embedded in an ethylcellulose film were recently demonstrated (72). An alternating magnetic field heated the nanoparticles, triggering collapse of the nanogel and increasing permeability of the membrane (**Figure 3**). This process was reversible, and numerous on and off cycles were demonstrated.

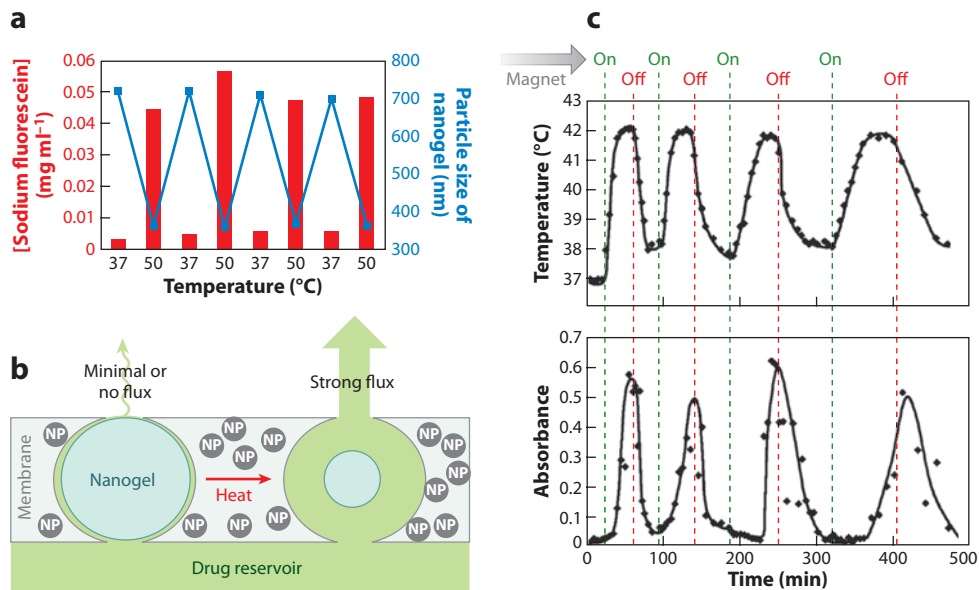


Figure 3

Stimulus-responsive membrane triggering. (a) Thermal triggering. Comparison of poly(*N*-isopropylacrylamide) nanogel particle size in suspension (blue data, right y axis) and differential flux of sodium fluorescein through nanogel-loaded membranes (red data, left y axis) as a function of temperature. (b) Schematic of the proposed mechanism of membrane function. NP denotes nanoparticles. (c) Magnetic triggering. Temperature profile in the sample chamber and differential flux of sodium fluorescein out of membrane-capped devices as a function of time over four successive on/off cycles of the external magnetic field. Adapted from Reference 72 with permission.

Ultrasound

Ultrasound is a longitudinal pressure wave that can trigger drug release by inducing either localized heating or mechanical disruptions (73). Ultrasound can be divided into two main categories: low-frequency ultrasound (LFUS), which ranges between ~20 and ~100 kHz, and high-frequency ultrasound (HFUS), which exceeds 1 MHz.

LFUS induces sonophoresis and affects drug carriers such as liposomes, polymeric micelles, and polymeric matrices and has also been used to enhance membrane permeability for drug and gene delivery. For example, LFUS was recently used to trigger cisplatin release from liposomes, a phenomenon that was attributed to a transient introduction of pore-like defects in the liposome membrane (74). Drug release from these structures effectively treats murine tumors (75). Moreover, microbubbles conjugated to liposomes can increase sensitivity to LFUS (76).

Ultrasound has been used to trigger the formation and collapse of cavitation bubbles and, depending on the amplitude of the wave, can produce high local pressures and temperatures. These phenomena have been demonstrated with HFUS, which has the benefit that it can be focused. Drug release has been triggered from microbubbles bearing more than 1,000 drug-containing liposomes each. Pulses were used first to push the microbubble carriers toward a cell monolayer and then to collapse the bubbles and release the liposomes (77). HFUS has also been used to release drugs from polymer micelles, most likely because of shear stress and shock waves formed by the collapse of cavitation bubbles (73). This phenomenon was demonstrated in the case of poly(lactic acid) and poly(ethylene glycol) (PEG) block copolymer (PLA-*b*-PEG)

micelles, which were used to encapsulate a model drug that was released upon application of HFUS (78).

Other Processes

Several other processes have been used to achieve drug release and warrant brief comment. UV and visible light have been used to trigger bond cleavage (79) or isomerization (80). Long-wavelength light such as radio-frequency or microwave radiation has also been used to heat gold nanoparticles and to dehybridize conjugated DNA (81) or to deform polyelectrolyte (82) or silica (83) microcapsules. Finally, electric fields have been used to release drugs from microfabricated reservoirs by oxidizing and dissolving a gold film sealing layer (84) or to destabilize and release drugs from redox-active layer-by-layer polyelectrolyte films (85).

DIFFERENT ROUTES OF DELIVERY

Scientists have attempted to deliver drugs through essentially every portion of the body. Although the oral route is the most common, scientists have delivered drugs to or through the nose, lung, vagina, uterus, skin, eye, vascular system, teeth, buccal membrane, and brain.

The oral route is still an area of very active research. For many years, a major challenge was the maintenance of constant drug release for a 24-h period. The development of approaches such as osmotic pumps has reduced this challenge, but other challenges remain. Foremost among these is the development of approaches for oral delivery of macromolecules such as proteins. One approach involves the development of carrier molecules, such as hydroxy-benzyl aminophenyl butyric acid, that carry large molecules across the epithelium in the gastrointestinal tract (86). Another approach involves the creation of bioadhesive microspheres that strongly adhere to the intestinal mucosa (87). A third approach involves the development of a bioadhesive patch that one can swallow, thereby enabling drug delivery through the intestinal area through which the patch adheres (88). Such an approach may also protect protein drugs from protease degradation.

The nasal route has also been widely studied. The concept of bioadhesion—discussed above for oral delivery—has also been used to prolong the residence time of molecules in the nose to enhance their bioavailability (89).

Similarly, there has been great interest in delivery to the lung. Although asthma drugs have commonly been delivered to the lung, an alluring goal is the possibility of delivering macromolecules such as insulin through this route. The FDA approved one such system (Exubera). But the large size of the inhaler, among other possible reasons, led to initially poor patient acceptance, and the marketer (Pfizer) removed it from the market. However, advances in improved aerosol design enable a much greater amount of aerosol to be delivered from the inhaler (see Reference 90 for an example). Such developments, in turn, have led to much smaller inhalers. It is hoped that such advances will lead to new approaches for pulmonary delivery.

Transdermal systems have been used clinically for fewer than 20 drugs or drug combinations. Nonetheless, they have had a great impact; several of these systems have reached sales of billions of dollars per year. Moreover, the types of drugs that can be delivered are greatly limited by their size and charge. Thus, extensive research is ongoing to explore methods of enhancing transdermal delivery. Examples include the use of chemical enhancers, electricity (iontophoresis, electroporation), ultrasound, and microneedles (91).

Controlled-release systems composed of polymeric microspheres that can be injected through the skin and deliver drugs, including peptides, from weeks to months are also widely used and continue to receive a great deal of attention. Such systems have led to new treatments for many diseases, including schizophrenia, alcoholism, and various forms of cancer (92).

Scientists have also attempted to explore different approaches to deliver drugs to the brain. Such systems include polymer matrices that slowly release anticancer drugs that can be directly placed in the brain at the time of brain tumor neurosurgery (which significantly increases patient survival probability) (93) as well as experimental studies in which drugs are conjugated to substances that are naturally transported across the blood-brain barrier (94).

Drugs have been delivered through both the intrauterine route and the vaginal route. In both cases, the intent has been for the delivery of low-molecular-weight drugs for improved contraception. Because of the enhanced permeability of the vagina, the possibility of delivering larger molecules may also be possible (95).

The eye was one of the first routes to be used for controlled drug delivery. In this area, polymer inserts, such as Ocusert, were used to deliver antiglaucoma drugs continuously for a week (as opposed to 28 eye drops) (96). Since that time, numerous other ocular systems for delivery to the front of the eye have been developed. However, with the recent advent of drugs that can now be used to treat macular degeneration, which occurs in the back of the eye, long-acting controlled-release systems that can be conveniently used in that site will become increasingly important.

Drugs have also been delivered to the vascular system to address serious medical problems. The development of implantable metal stents led to a major advance in propping open arteries for patients with heart disease. However, it also led to significant proliferation of smooth muscle cells at that site, leading to closing off of the blood vessel. This problem has been addressed by coating the metal with polymers containing antiproliferative drugs (e.g., PTX, rapamycin) in which the drug is slowly released locally over time, greatly reducing cell proliferation (97). Such drug-eluting stents—first approved by the FDA in 2003—have been used in millions of patients.

The buccal membrane, with its increased permeability, has been used for the delivery of nitroglycerin and pain medications. However, the possibility of using the buccal membrane for the delivery of large molecules such as insulin is appealing, and clinical trials are under way (98). Even our teeth and gums can be sites for drug delivery. The use of polymers to locally deliver drugs to treat periodontal disease has been widely studied (99).

TARGETED NANOPARTICLES FOR DRUG DELIVERY

In cancer therapy, nanoparticles that encapsulate therapeutic agents, including small-molecule drugs, peptides, proteins, and nucleic acids, have shown improved therapeutic efficacy with reduced side effects (100). The benefit is attributed in part to the preferential accumulation of nanoparticles through passive targeting via the leaky tumor vasculature and the well-known enhanced permeability and retention (EPR) effect (**Figure 4a**) (101). It has been hoped that the therapeutic outcome can be further improved through the modification of nanoparticle surfaces with ligands that bind to surface receptors on target cells. This alternative approach, known as active targeting, has triggered intensive preclinical studies and has resulted in targeted nanoparticles using a wide variety of ligands, including small molecules, antibodies, peptides, and aptamers (102).

In contrast to a plethora of targeted nanoparticles developed in preclinical studies, none of the current clinically approved nanoparticle drugs adopt active targeting (103). Although three targeted nanoparticle systems are now in phase I/II clinical trials, the majority of nanoparticle formulations that are currently under clinical development lack active targeting (104). In fact, nearly 30 years after the first examples of targeted liposomes were described in the literature (105, 106), targeted nanoparticles have not made a significant clinical impact on human health. Some doubt has been raised as to whether using targeting ligand in nanoparticle drugs is a luxury or a

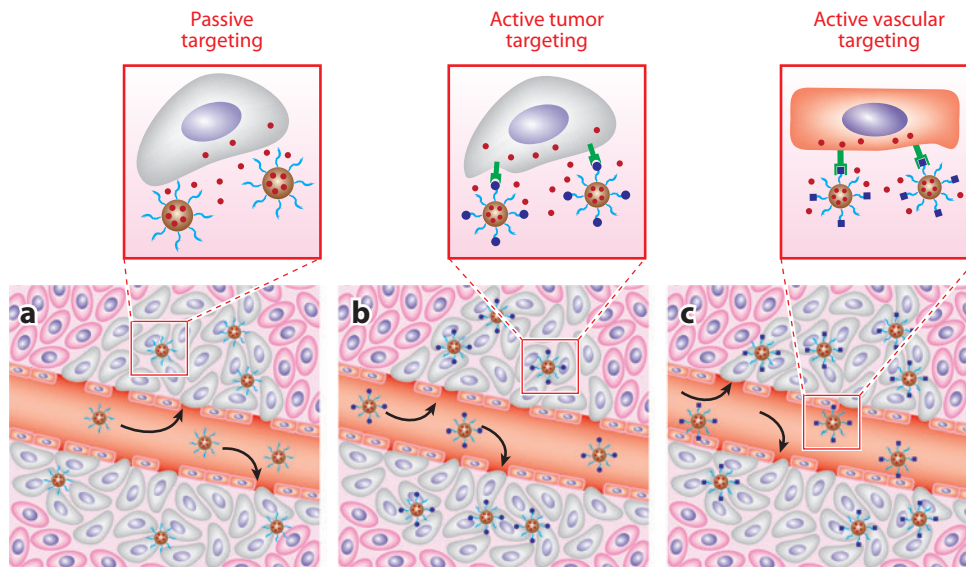


Figure 4

Passive versus active targeting. (a) Particles tend to passively extravasate through the leaky vasculature, which is characteristic of solid tumors and inflamed tissue, and to preferentially accumulate through the enhanced permeability and retention effect. In this case, the drug may be released in the extracellular matrix and may diffuse throughout the tissue for bioactivity. (b) Once particles have extravasated in the target tissue, the presence of ligands on the particle surface can result in active targeting of particles to receptors that are present on the target cell or tissue, resulting in enhanced accumulation and cell uptake through receptor-mediated endocytosis. This process, referred to as active targeting, can enhance the therapeutic efficacy of drugs, especially those that do not readily permeate the cell membrane and that require an intracellular site of action for bioactivity. (c) The particles can be engineered for vascular targeting by incorporating ligands that bind to endothelial cell-surface receptors. Although the presence of leaky vasculature is not required for vascular targeting, when such vasculature is present, as in the case in tumors and inflamed tissue, this strategy may work synergistically for drug delivery to target both the vascular tissue and target cells within the diseased tissue for enhanced therapeutic efficacy.

necessity. The answer is complex and can be found only by investigating the roles of targeting ligand in determining nanoparticle therapeutic efficacy on a case-by-case basis. Nevertheless, some considerations in developing targeted nanoparticles can still be generated on the basis of diseases and indications, the choices of drugs and targeting ligands, and the engineering principles of delivery vehicles.

Although targeted nanoparticles have been developed for a myriad of important diseases, the thrust of research has been focused on solid tumors, cardiovascular diseases, and immunological diseases. As mentioned above, the currently approved nanoparticle drugs for cancer therapy function by accumulating in tumor tissue through the EPR effect and by releasing their payload in the extravascular tumor tissue for antitumor efficacy. Although these nontargeted nanoparticles have been clinically efficacious, there have been contradicting data regarding the added benefit of the inclusion of targeting molecules to these systems (107–110).

Tumor tissue accumulation is a passive process requiring a long circulating half-life to facilitate time-dependent extravasations of nanoparticles through the leaky tumor microvasculature and the accumulation of drugs in the tumor tissue. This process is strongly dependent on the biophysicochemical properties of the nanoparticles, but not on active targeting. Therefore, even

in the absence of targeting ligands, nontargeted nanoparticles can still be engineered to better target a particular tissue, or be nonspecifically absorbed by cells, by optimizing their biophysicochemical properties. However, as illustrated in **Figure 4b**, once particles extravasate out of the vasculature into the tumor tissue, their retention and specific uptake by cancer cells are facilitated by active targeting and receptor-mediated endocytosis, which lead to higher intracellular concentration and, in some cases, increased cellular cytotoxicity of small-molecule drugs. Ligand-mediated cell internalization becomes especially crucial for delivering bioactive macromolecules such as DNA and siRNA, which rely on intracellular delivery for bioactivity. Studies using similar nanoparticles containing cyclodextrin, PEG, and amine-containing polycations to deliver siRNA (111), plasmid DNA, and a DNAzyme (110) showed consistent results that the amount of nucleic acid-reaching tumors did not depend on the presence of transferrin used as the target ligand; however, intracellular localization of nucleic acids and expression within tumor cells occurred only when transferrin presented. Similarly, by use of nanoparticles as a vaccine for immunological diseases, ligands are crucial in targeting dendritic cells, especially in peripheral tissues such as skin and muscle, where dendritic cells reside in much lower numbers (112). Nanoparticle size strongly influences nanoparticle transport through the lymphatic vessels to draining lymph nodes. Through use of pluronic-stabilized polypropylene sulfide nanoparticles, smaller-sized (~25-nm) nanoparticles resulted in more efficient antigen presentation to dendritic cells in the lymph nodes compared with larger (~100-nm) nanoparticles (113).

In the case of vascular endothelial targeting for oncology or cardiovascular indications, because tissue accumulation is not a function of EPR, ligand-mediated targeting is indispensable to ensure therapeutic efficacy (**Figure 4c**) (114). For example, in studies of nanoparticle drug delivery to atherosclerotic plaques (115) and the basement membrane of angioplasty-injured vasculature (116), substantial nanoparticle accumulation was found when small peptides were conjugated to nanoparticle surfaces as targeting ligands; however, the absence of targeting ligand resulted in only negligible accumulation of nontargeted nanoparticles. Additionally, in vascular endothelial targeting, ligand-receptor binding affinity strongly affects the accumulation of targeted nanoparticles at pathological tissues. As a result, a number of studies have been devoted to the search of high-affinity targeting ligands (116–118).

The rapid development of nanoparticle drug delivery has been accompanied by an increasing effort to identify new disease biomarkers and associated ligands for use in targeted nanoparticle drug delivery (119). For example, a variety of small molecules have been widely used for this purpose owing to their small size, low cost, and robustness. Despite these advantages, they usually lack specificity in targeting. To overcome this limitation, monoclonal antibodies have become popular targeting ligands with significantly improved specificity. However, their large and complex molecular structure and high production cost hinder their wide application in targeted nanoparticles. In contrast, antibody fragments retain the high antigen-binding specificity of antibodies, whereas their smaller molecular size results in enhanced tissue penetration and ease of engineering. As another class of targeting ligands, short peptides have also been increasingly used in targeted nanoparticles and show further improved specificity and affinity. Additional advantages include their small size, low immunogenicity, high stability, and ease of manufacturing. The development of screening technologies using peptide phage libraries, bacterial peptide display libraries, and plasmid peptide libraries has made the selection of short peptides much easier, contributing to their popularity as targeting ligands. Similar to short peptides, aptamers are single-stranded nucleic acids (of ~15 kDa), which fold into unique conformations that can bind to targets with superior affinity and specificity. With the development of systemic evolution of target-specific ligands by exponential enrichment (SELEX), a screening technique that depends on the progressive selection of highly specific ligands by repeated rounds of partition and amplification from a large

combinatorial nucleic acid library, more than 200 aptamers have now been isolated and can be useful as targeting ligands in nanoparticle drug delivery.

Understanding the role of these targeting ligands in specific diseases or indications can guide the choice of drugs for targeted nanoparticle delivery because the optimal efficacy can be achieved only when the mechanism of drug acting on the disease and the interaction of ligand with pathological cells or tissues are mutually compatible or even synergistic. For example, the delivery of therapeutics with intracellular sites of action and inefficient cellular uptake may be best achieved with targeted nanoparticles. Examples include the RNAi or antisense therapeutics mentioned above. Conversely, if a therapeutic requires intracellular delivery for bioactivity, then therapeutic efficacy may require homogeneous tissue penetration and cellular uptake of the targeted drug delivery vehicle, which is difficult to achieve (120). In some cases, targeting may anchor drug delivery systems and may decrease the efficiency of diffusion and uniform tissue distribution. The optimization of the ligand density on the nanoparticle surfaces can be a potential solution to facilitate the balance between tissue penetration and cellular uptake and to lead to optimal therapeutic efficacy. The targeting of cell-surface receptors that participate in membrane recycling pathways facilitates the uptake of targeted drug delivery systems through receptor-mediated endocytosis. Interestingly, in some cases, distinct cell-surface receptors are responsible for nanoparticle attachment and internalization (121), which may provide useful tools to precisely control nanoparticle cell uptake by tailoring ratio, surface density, and distribution of different ligands on the same nanoparticle. In addition, understanding endosomal trafficking pathways, which are complex and can vary among receptors, can facilitate the engineering of suitable targeted nanoparticles. For example, the choice of targeting ligand used and hence the endocytic pathway taken influence the extent of endosomal acidification. The pH of early endosomes, sorting endosomes, and multivesicular bodies can drop rapidly to pH 6.0 and below, allowing nonrecycling ligands such as low-density lipoproteins to dissociate from their receptors and to sort into late endosomes for degradation. In contrast to such nonrecycling receptors, trafficking of recycling receptors is thought to proceed through a distinct sorting compartment—the recycling center, which is characterized by near-neutral pH values between 6 and 7—before returning to the plasma membrane for reuse (122). Folate leads to endocytosis through recycling centers characterized by near-neutral pH values, which may therefore make it a better ligand for delivering pH-sensitive drugs but less suitable for mechanisms relying on acidic pH (123).

For the successful development and manufacturing of targeted nanoparticles, the compatibility of targeting ligands with other biomaterials and engineering processes should be evaluated and optimized. Additional considerations for engineering principles of delivery vehicles include (*a*) the use of biocompatible materials to achieve simple but robust processes for conjugation chemistry, purification steps, and nanoparticle assembly; (*b*) the ability to optimize in parallel the myriad of biophysicochemical parameters of targeted nanoparticles important for pharmacokinetic properties and possible cell uptake; and (*c*) the development of scalable unit operations amenable to manufacturing large quantities of targeted nanoparticles needed for clinical translation. Currently, most formulation processes require multiple steps for biomaterial assembly, ligand attachment, and purification. The resulted batch-to-batch variation and quality concerns inevitably impose on the wide translation of targeted nanoparticles. The recent development of targeted nanoparticles by self-assembly of prefucionalized biomaterials simplifies the optimization and the potential manufacturing of these systems (124, 125). These studies also show that the nature and density of the ligands on nanoparticle surfaces and their subsequent effects on the biophysicochemical properties of the particles, such as size, charge, surface hydrophilicity, and geometry, can all profoundly impact the circulating half-life of the particles and their biodistribution. For example, the presence of targeting ligands can increase the interaction of the drug delivery system with a subset

of cells in the target tissue, which can enhance cellular uptake by receptor-mediated endocytosis. Surface properties of nontargeted drug delivery vehicles such as ordered striations of functional groups as well as their shape and size also enhance particle uptake (126). More recently, scientists found that the attachment of two different targeting ligands to the same nanoparticle induced potent cytotoxicity; however, no obvious cytotoxicity was observed if only one type of ligand was conjugated (127).

The enormous complexity in nanoparticle optimization combined with the urgent need to develop efficacious nanoparticle drugs have accelerated the development of high-throughput technologies to meet the requirements. For example, particle replication in nonwetting templates (PRINT) allows for rapid fabrication of nanoparticles in which a single parameter such as shape or size can be precisely altered independently of all other particle attributes (128). Microfluidic rapid mixing can synthesize targeted nanoparticles with variable components and reproducible monodispersity using a minimum number of preparation steps (129). High-throughput synthetic methods have enabled rapid synthesis of large libraries of structurally diverse materials capable of subsequent functional screening (130).

In summary, these general considerations in developing targeted nanoparticles indicate that targeting ligand plays a critical role in determining nanoparticle therapeutic efficacy and is a crucial component in engineering nanoparticle drug delivery. With continuing studies in targeting ligand and nanoparticle optimization, targeted nanoparticles will be a mainstay in the next generation of drug therapy.

CONCLUSIONS

We discuss above four areas of great interest in the field of drug delivery. Research in each of them is extremely active and promises to increase in the future. This is for a good reason, as these approaches enable drugs and biopharmaceuticals to be much safer, more convenient, and more effective. In many cases, they either are enabling or will enable entirely new life-saving and life-enhancing medical therapies.

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