Extracellularly Activated Nanocarriers: A New Paradigm of Tumor Targeted Drug Delivery

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Abstract: One of the main goals of nanomedicine is to develop a nanocarrier that can selectively deliver anticancer drugs to the targeted tumors. Extensive efforts have resulted in several tumor-targeted nanocarriers, some of which are approved for clinical use. Most nanocarriers achieve tumor-selective accumulation through the enhanced permeability and retention effect. Targeting molecules such as antibodies, peptides, ligands, or nucleic acids attached to the nanocarriers further enhance their recognition and internalization by the target tissues. While both the stealth and targeting features are important for effective and selective drug delivery to the tumors, achieving both features simultaneously is often found to be difficult. Some of the recent targeting strategies have the potential to overcome this challenge. These strategies utilize the unique extracellular environment of tumors to change the long-circulating nanocarriers to release the drug or interact with cells in a tumor-specific manner. This review discusses the new targeting strategies with recent examples, which utilize the environmental stimuli to activate the nanocarriers. Traditional strategies for tumor-targeted nanocarriers are briefly discussed with an emphasis on their achievements and challenges.

Keywords: Nanocarriers; tumor targeting; passive targeting; active targeting; activatable (activated) nanocarriers; drug delivery

Introduction

One of the main goals of nanomedicine is to develop a safe and effective drug carrier that is systemically applied but will selectively deliver cytotoxic drugs to tumor cells without harming normal cells. The unique structural features of many solid tumors (hypervasculature, defective vascular architecture, and impaired lymphatic drainage)[1,2] lead to relatively selective extravasation and retention of long-circulating nanocarriers. This phenomenon ("passive targeting") is essentially the working principle of most clinically viable targeting strategies based on nanocarriers.

Another popular approach is to modify the surface of nanosized carriers with ligands that can specifically recognize the tumor cells ("active targeting"). This strategy relies on specific interactions between the ligands (antibodies, peptide mimics, or nucleic acids) on the carrier surface and receptors expressed on the tumor cells. For example, human epidermal growth factor receptor-2 (HER-2),[3–5] folic acid receptor,[6]

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and vasoactive intestinal peptide receptors (VIP-R)[7] have been investigated as biomarkers for nanocarriers targeted to breast tumors.

Recently, new targeting strategies have emerged as a way of improving the targeting efficiency of the nanocarriers. These strategies utilize the unique microenvironment surrounding tumor cells ("tumoral extracellular environment") as a molecular cue to activate long-circulating nanocarriers to release the drug or facilitate their cellular uptake upon arrival at the target tumor sites.

The objective of this review is to discuss recent advances in tumor targeting strategies employed in nanocarrier-based drug delivery, with an emphasis on extracellularly activated nanocarriers. Traditional targeting strategies will be briefly reviewed first, and new targeting strategies based on the tumoral extracellular environment will subsequently be discussed with recent examples.
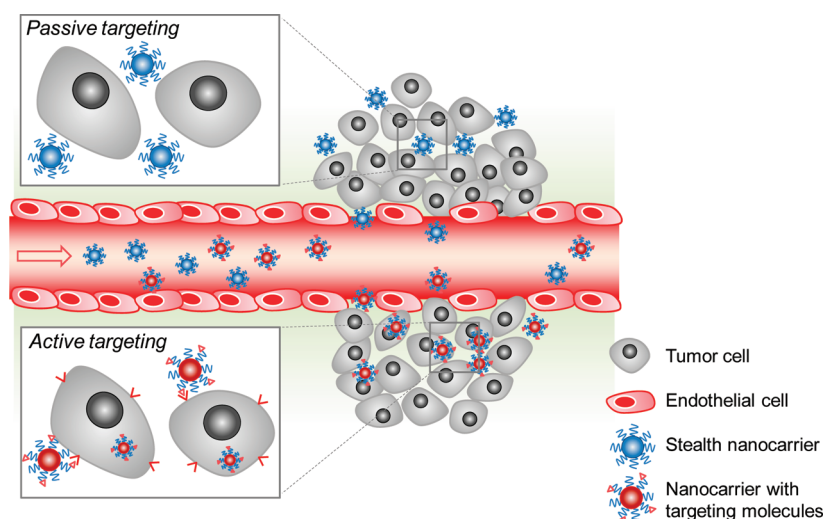
Nanocarriers

Many different macromolecular structures, such as drug—polymer conjugates, micelles, liposomes, dendrimers, and nanoparticles, have been designed to transport drugs to their intended targets. Micelles can be made from amphiphilic block-copolymers that self-assemble into small spherical structures. [8−10] Liposomes are vesicles made of lipid bilayers that can encapsulate drugs in their cores or bilayers.[10] Nanoparticles are generally polymeric matrix in the form of nanosized colloids that can encapsulate a drug through physical entrapment (association between the drug and polymer) or chemical conjugation (creating a chemical bond between the drug and polymer).[11,12] For the sake of brevity,

all these systems will be referred to as nanocarriers unless a specific design is the focus. Recent reviews provide detailed information about various types of nanocarriers.[10,12−24]

Traditional Targeting Strategies

This section briefly summarizes the traditional targeting strategies focusing on their achievements and limitations. Interested readers are referred to recent review articles that discuss each strategy more comprehensively.[10,14,15,18,21,22,25−31]

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Figure 1. Schematic representation of nanocarriers that passively or actively target tumors. Both types of nanocarriers reach tumors selectively through the leaky vasculature surrounding the tumors. Upon arrival at tumor sites, nanocarriers with targeting molecules can bind to the target tumor cells or enter the cells via specific receptor (cell)−ligand (carrier) interactions, whereas stealth nanocarriers are less efficient in interacting with tumor cells.

Passive Targeting. One of the most useful discoveries in tumor physiology for tumor-targeted drug delivery is the enhanced permeability and retention (EPR) effect.[1,32,33] An actively growing tumor needs to form new blood vessels in order to get nutrients and sustain its growth.[13] These newly formed blood vessels are usually defective and have fenestrae ranging in size from 100 to 600 nm.[13,34−36] The targeting effect is achieved as the nanocarriers remain in the bloodstream, where the vessel structure is normal, but would extravasate through the leaky vasculature at the tumor site (Figure 1). Upon arrival at the target sites, the nanocarriers release the drug at the vicinity of the tumor cells.[37] The impaired lymphatic drainage in tumors contributes to retention of the nanocarriers at the tumors.[13]

To take advantage of the EPR effect, it is critical for the nanocarriers to evade immune surveillance and circulate for a prolonged period. To this end, at least three properties of nanocarriers are particularly important. Most gaps in the leaky vasculature fall below 400 nm;[36] therefore, nanocarriers should be much less than 400 nm for efficient extravasation. On the other hand, the kidneys are capable of filtering particles smaller than 10 nm (about 70,000 Da),[38,39] and the liver can capture particles larger than 100 nm.[39] Therefore, the ideal nanocarrier size is somewhere between 10 and 100 nm. Not only can clearance organs filter out nanocarriers based on their size, the kidneys are also capable of filtering positively charged particles.[40,41] Therefore, it is important to maintain the nanocarriers either neutral or anionic for successful evasion of the renal elimination. In addition, the nanocarriers must be hidden from the reticuloendothelial system (RES), which destroys any foreign material through opsonization followed by phagocytosis.[16,42] A common method of disguising nanocarriers from the RES is to coat the surface with polyethylene glycol (PEG), a procedure called PEGylation.[14,42,43] The most widely accepted theory for this increased circulation time is that PEG reduces the protein interactions on the surface of the nanocarriers and

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prevents their binding to opsonins.[42,44] This may be due to the hydrophilicity of PEG and the attraction of water to the surface which repels the protein.[16,44] One study showed that the half-life of gelatin nanoparticles increased from 3 to 15 h when they were PEGylated.[16]

A number of passively targeting nanocarriers were developed in the 1980s and 1990s,[10,45] and some of them were approved for clinical use.[46] One of the examples is Doxil (or Caelyx), a PEGylated liposome that encapsulates doxorubicin. Doxil/Caelyx has shown enhanced circulation time compared to free doxorubicin, good drug retention in the liposomes[46] and is up to six times more effective than free doxorubicin.[36,46,47] Doxil/Caelyx was approved for advanced ovarian cancer, metastatic breast cancer, and AIDS-related Kaposi's sarcoma.[18]

While the long-circulating nanocarriers significantly increased tumor localization of the payload, some limitations exist. First, the targeting effect is highly dependent on the degree of tumor vascularization and angiogenesis.[13] The porosity and pore size of tumor vessels vary with the type and status of the tumors;[48,49] thus, the passive targeting effect may not be always achieved in all tumors. Second, the elevated interstitial fluid pressure, a condition common to most solid tumors,[50] inhibits efficient uptake and homogeneous distribution of nanocarriers and/or drugs in the tumor tissues.[13,51] Moreover, the passive targeting can be further limited due to the very presence of PEG. The PEGylated surface can prevent not only the interaction between the nanocarriers and opsonins but also that between the nanocarriers and cell surface.[52−56] The reduced interactions inhibit effective uptake of the payload by the tumor cells.[57] For example, PEGylated liposomal doxorubicin was less efficient

in entering the tumor cells, with a much lower AUC_{tumor}/AUC_{plasma} (0.31) (AUC: area under the curve) compared to that of non-PEGylated liposomes (0.87).[55] The reduced cellular uptake of PEGylated carriers would be particularly problematic in drug delivery to multidrug-resistant tumor cells, as the drug is released in the extracellular matrix and enters the cells as a free drug, which will be subjected to the multidrug resistance machinery.[24,48]

Active Targeting. Active targeting involves conjugating targeting molecules to the surface of nanocarriers. Examples of targeting molecules include antibodies, ligands, peptides, nucleic acids, and other molecules that bind directly to a receptor overexpressed on a tumor-cell surface.[16] The idea of active targeting was first proposed by Paul Ehrlich in the 19th century, even before a rational targeting ligand was discovered. Ehrlich coined the term "magic bullet", an idealized package that would target and deliver drugs to a specific place in the body.[58] This idea translates directly to the ongoing effort in cancer research to reduce the systemic toxicity of chemotherapy. The underlying rationale of active targeting is that the avid and specific interaction between the nanocarriers and the target cells would selectively increase the rate and extent of drug delivery to the target tumor cells.[10,16,18,36] The endothelium surrounding tumors, which does not necessarily express the tumor-specific target molecules, may be a potential barrier to the direct interaction between the nanocarriers and tumors. Nonetheless, the ligands are expected to have direct access to the target molecules expressed on the underlying tumor tissues and achieve the active targeting effect, because the

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vascular system at tumor sites does not have a normal barrier function due to the abnormal structure.[59]

The selective and efficient drug delivery by the actively targeting nanocarriers has been demonstrated in numerous studies *in vitro* and/or *in vivo*. Table 1 summarizes selected examples showing the effectiveness of the active targeting strategy. A variety of targeting molecules have been chosen to demonstrate the multitude of molecular options. These results show that active targeting nanocarriers can more effectively bind, enter, and kill tumor cells than nontargeted nanocarriers.

Since targeting molecule−drug conjugates achieve high levels of biodistribution in tumors,[60−62] the same may be expected for the targeted nanocarriers.[10,63] Recent studies

report mixed results. A number of studies found that the targeting ligands did not improve the tumor disbribution of nanocarriers.[4,5,36,64−67] A study comparing immunoliposomes and nontargeted liposomes showed that biodistribution of the two liposomes was similar.[5] Epidermal growth factor receptor (EGFR)-targeted immunoliposomes were injected intravenously to mice bearing EGFR-overexpressing tumors, and their biodistribution was compared with that of PEGylated (nontargeted) liposomes.[5] The antibodies did not increase the amount of liposomes at the tumor site nor did it shorten the time for the nanocarriers to reach the target tissues.[5] Another example shows that HER2-targeted immunoliposomes had no difference in biodistribution or tumor accumulation time versus nontargeted liposomes.[4] Bartlett et al. reported a similar observation.[64] Transferrin receptor (TfR)-targeted nanoparticles were created by labeling transferrin to nanoparticles consisting of cyclodextrin-containing

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Table 1. Examples of Nanocarriers Employing the Active Targeting Strategy

platform	targeting molecule	results
PEGylated liposomes	nucleosome-specific monoclonal antibody 2C5[63,68,107,108]	*In vitro*: Targeted liposomes encapsulating doxorubicin showed 2.3 times (in murine Lewis lung carcinoma cell line) or 1.6 times (in human mammary adenocarcinoma cell line) higher cytotoxicity than nonspecific IgG-modified liposomes at a doxorubicin level of 100 μg/mL.[107] The IC_{50} values of targeted liposomes in various murine and human cell lines were 5−8-fold lower than those of control liposomes (PEGylated or modified with nonspecific IgG).[108] *In vivo*: Targeted liposomes showed 2−3 times higher tumor accumulation than nonspecific IgG-conjugated or plain liposomes in nude mouse models of murine Lewis lung carcinoma (LLC)[63] or murine breast adenocarcinoma,[68] by whole body gamma-scintigraphic imaging. Antitumor activity of the targeted liposomes (with doxorubicin) was superior to those of plain or nonspecific IgG-conjugated liposomes in LLC model,[63] murine breast adenocarcinoma, murine colon cancer, and human prostate cancer models.[68]
PEGylated liposomes	anti-HER2 monoclonal antibody fragments[4,109]	*In vivo*: Both targeted and nontargeted liposomes achieved similar levels of tumor tissue accumulation in a nude mouse model of HER2-overexpressing breast cancer (BT-474), measured by radioactivity counting.[4] Targeted liposomes were found within tumor cells, whereas nontargeted ones were predominantly in extracellular stroma and macrophages. Targeted liposomes were found in the extracellular stroma and macrophages in non-HER2-overexpressing breast cancer (MCF-7) model. Targeted liposomes containing doxorubicin achieved superior antitumor efficacy over nontargeted liposomes.[109]
PEGylated liposomes	thiolated herceptin[3,70]	*In vitro*: Targeted liposomes achieved higher cellular uptake than plain PEGylated liposomes in HER2-overexpressing breast cancer cell lines (SK-BR-3, and BT-474) but not in low HER2-expressing cells (MDA-MB-231).[3,70] *In vivo*: Targeted liposomes containing paclitaxel showed a higher ratio of tumor to plasma drug concentrations (T/P ratio) than nontargeted liposomes in HER2-overexpressing breast carcinoma model (BT-474) but not in low HER2-expressing model (MDA-MB-231).[70] Targeted liposomes containing paclitaxel achieved higher antitumor efficacy than the nontargeted liposomes in the BT-474 model.[70]
PEGylated liposomes	folate[65]	*In vivo*: Targeted liposomes showed similar tumor accumulation as nontargeted liposomes in BALB/c mice with high folate receptor (FR)-expressing tumors (mouse M109, human KB carcinomas), measured by radioactivity counting.[65]
PEGylated liposomes	epidermal growth factor receptor (EGFR)[5]	*In vivo*: Total tumor accumulations of targeted and nontargeted liposomes were similar in a nude mouse model of EGFR-overexpressing MDA-MB-468 tumor, measured by radioactivity counting. Targeted liposomes internalized in the tumor cells efficiently (92% of analyzed cells) unlike nontargeted liposomes (<5%) in a nude mouse model of U87 glioblastoma (EGFR variant). Targeted liposomes (containing doxorubicin, epirubicin, or vinorelbine) achieved higher antitumor effects than nontargeted liposomes.[5]
PEGylated liposomes	Arg-Gly-Asp (RGD) peptide[66,67]	*In vivo:* Targeted liposomes and nontargeted liposomes showed similar drug accumulation in tumors in a nude mouse model of murine B16 melanoma.[66,67] Targeted liposomes containing doxorubicin had higher antitumor effect than nontargeted liposomes.[66,67]
PLGA nanoparticles	monoclonal antibody to cytokeratin of MCF-7 human breast cancer cells[110]	*In vitro*: Targeted nanoparticles entered specifically to MCF-10A neoT human breast epithelial cells, in a coculture of MCF-10A neoT and Caco-2 cells, whereas nontargeted particles were distributed randomly.[110]
PLGA nanoparticles	folate[111]	*In vitro*: Targeted nanoparticles entered KB (FR- overexpressing) more efficiently than nontargeted particles.[111]
PLGA nanoparticles	prostate-specific membrane antigen (PSMA)-specific aptamer[72,75,76,112]	*In vitro*: Targeted nanoparticles entered in LNCaP (PSMA-expressing) but not PC3 (PSMA-deficient) prostate cancer cells.[75,112] *In vivo*: When optimally formulated, targeted nanoparticles achieved higher tumor accumulation than nontargeted particles in a BALB/c mouse model of LNCaP prostate cancer.[76,77]
poly(ε-caprolactone)-poly(ethyl ethylene phosphate) micelle	galactosamine[113]	*In vitro*: Targeted micelles showed superior cell binding, uptake, and paclitaxel delivery over the HepG2 (asialoglycoprotein receptor-expressing) cells.[113]
poly(lactic acid)-poly(ethylene oxide) micelle	Arg-Gly-Asp (RGD) peptide[69]	*In vivo*: Targeted micelles showed higher tumor accumulation than nontargeted micelles in a nude mouse model of MDA-MB-435 breast tumor, measured by radioactivity counting. Targeted micelles carrying paclitaxel were more effective in tumor regression than nontargeted micelles.[69]
poly(D,L-lactic-*co*-glycolic acid)-poly(ethylene glycol) micelle	folate[71,114]	*In vivo*: Targeted micelles carrying doxorubicin showed higher drug concentration in tumor and higher antitumor effect than nontargeted micelles in a nude mouse model of KB human squamous cell carcinoma.[71]
cyclodextrin-containing polycations and siRNA	transferrin[64]	*In vivo*: Both targeted and nontargeted nanoparticles exhibited similar biodistribution and tumor localization by PET in an immunodeficient mouse model of Neuro2A-Luc cell tumor. Targeted nanoparticles showed 50% higher gene silencing in tumor than nontargeted ones.[64]

polycations and small-interfering RNA (siRNA).[64] Positron emission tomography (PET) of ^{64}Cu-labeled nanoparticles showed that both nontargeted and targeted exhibited similar biodistribution and tumor localization.[64] These observations indicate that the biodistribution of the targeted nanocarriers in tumors is mostly governed by the EPR effect rather than
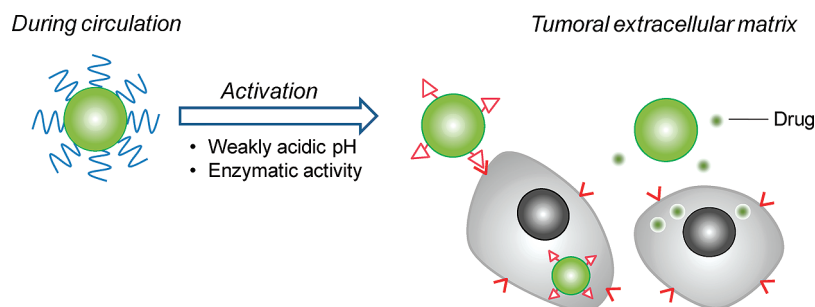
the interaction between the targeted nanocarriers and the target cells. In line with this interpretation, Gabizon et al. showed that coadministration of free folate had a negligible effect on the tumor deposition of folate receptor (FR)-targeted liposomes,[65] suggesting that the interaction of the liposomes with tumor cells did not play a critical role in their biodistribution. On the other hand, Torchilin et al. recently reported that the nucleosome-targeted immunoliposomes showed 2−3 times higher tumor accumulation than nonspecific IgG-conjugated or plain liposomes in murine carcinoma models using the whole body gamma-scintigraphic imaging.[63,68] Several other groups have also reported higher tumor distribution of targeted nanocarriers as compared to nontargeted ones.[69−71]

Notably, the former group of studies suggests that the targeting molecules play a role *after* the nanocarriers are distributed in the tumor tissues. Although the tumor distributions of targeted and nontargeted nanocarriers were similar, only targeted nanocarriers could efficiently enter the tumor cells from the extracellular space (Figure 1). Studies using colloidal gold-labeled liposomes show that the HER2-targeted immunoliposomes accumulated within tumor cells, whereas nontargeted liposomes were located predominantly in the extracellular matrix.[4] Similarly, the extent to which the EGFR-targeted immunoliposomes were found inside the tumor cells was 6-fold higher than that of nontargeted liposomes.[5] In another example, where FR-targeted liposomes were injected intravenously to mice with ascitic lymphoma, the overall accumulation of FR-targeted liposomes in ascites was somewhat lower than that of the nontargeted ones, but the fraction of FR-targeted liposomes associated with tumor cells was much higher compared to nontargeted liposomes.[42] The increased cellular uptake of targeted nanocarriers was also demonstrated with the TfR-targeted nanoparticles carrying siRNA.[64] Cellular uptake of the nanoparticles was estimated from the gene-silencing effect of the siRNA. The activity of reporter gene product (luciferase) in mice treated with TfR-targeted nanoparticles was 50% lower as compared to nontargeted nanoparticles, indicating more efficient entry of the targeted nanoparticles into the tumor cells.[64] In light of these results, the difference between targeted and nontargeted nanocarriers in tumor distribution observed by other studies[63,68−71] can be interpreted alternatively. The superior tumor accumulation of targeted nanocarriers may be another reflection of their efficient entry to the tumor cells *following* extravasation. A study comparing antitumor effects of intratumorally injected nanoparticles implies that the nontargeted particles could be cleared from the tumor sites unless they were subsequently taken up by the cells.[72] Since both targeted and nontargeted nanoparticles were directly injected to the tumors, their initial distributions in tumors would have been comparable. However, the nontargeted nanoparticles were inferior to the targeted ones in antitumor effect, suggesting that the nontargeted particles were cleared from the tumor sites due to the lack of cellular uptake.[72] Therefore, the difference between targeted and nontargeted nanocarriers in tumor

distribution[63,68−71] may be viewed as a result of efficient cellular entry and intracellular retention of the targeted nanocarriers rather than tissue-specific distribution of the carriers. Here, the role of the targeting ligand would be to prevent potential clearance of the nanocarriers (e.g., re-entry to the bloodstream) from the tumor tissues rather than to increase "recognition" of the targeted sites during circulation.

The active targeting strategy is also not without limitations. First, the targeting molecules can expose the nanocarriers to the RES system during the circulation.[3,63,65,68,73,74] Aptamers, or small fragments of RNA or DNA, have been used as a targeting molecule due to their small size and lack of immune response.[75] Aptamers fold into shapes that induce high binding specificity to their target molecules.[75] An *in vitro* study shows high nanoparticle uptake in targeted cells, approximately 40%, when the nanoparticles were conjugated to the aptamers.[76] Cells without the specific antigen showed very little nanoparticle uptake, approximately 5%.[76] Particles with nonfunctional aptamer showed ∼5% uptake whether the cells express the specific antigen or not.[76] However, *in vivo*, the nanoparticles showed very little tumor accumulation compared with accumulation in the liver and spleen.[76] Only 1.5−2% of injected dose per gram (IDPG) accumulated at the tumor site while 30−60% of IDPG accumulated in the liver.[76] The accumulation in the spleen ranged from 10% to 30%.[76] Another study showed significant accumulation of aptamer-conjugated PEG/PLGA nanoparticles in the spleen.[77] The IDPG in the spleen was over 60% while the tumor accumulation was only approximately 2%.[77] The high accumulation of the nanocarriers in the RES organs (liver, spleen) would be undesirable unless they are the intended targets, because the anticancer drugs may potentially damage the RES organs and/or be destroyed before they reach the tumor. Second, while the long circulation time is critical for selective distribution of the nanocarriers at the tumor sites, the recognition of the targeted nanocarriers by the RES expedites clearance of the nanocarriers during circulation.[3,45,63,65−68] The addition of antibodies on the surface appears to compromise the shielding effect of the PEG layer.[36] These limitations may partly explain the lack of active targeting nanocarriers currently approved by the FDA.[18]

Active targeting strategy improves the anticancer effect of a drug by facilitating cellular uptake and intracellular retention of the drug carriers. In particular, the active targeting strategy is a promising tool for overcoming the multidrug resistance, for which the passively targeted nanocarriers do not much avail,[48] as the actively targeted nanocarriers can provide an intracellular drug reservoir. On the other hand, tumor distribution of the targeted nanocarriers is largely governed by the same principle as nontargeted nanocarriers (the EPR effect) and the targeting molecules do not seem to play a role until the carriers reach the target tissues. Therefore, an ideal nanocarrier should attain both the EPR effect and the specific and avid interactions between the targeted nanocarriers and tumors, which will lead to maximum tumor distribution and cellular uptake of the

During circulation   *Tumoral extracellular matrix*

Activation
- Weakly acidic pH
- Enzymatic activity

Drug

Figure 2. Schematic representation of an "extracellularly activated nanocarrier." The nanocarrier maintains the stealth function during circulation (passive targeting). Upon arrival at the tumor sites, the nanocarriers transform to release the drug or interact with cells in a target-specific manner (active targeting). Such transformation can be triggered by the unique tumoral extracellular environment such as slightly acidic pH or a high level of proteinases.

nanocarriers, respectively. To this end, it is critical to design a nanocarrier system that is "maximally targeted and maximally stealth."[76]

Tumor Targeting via Extracellular Activation of Nanocarriers: A New Paradigm of Tumor-Targeted Drug Delivery

Some of the recently reported targeting strategies have good potential to achieve both passive and active targeting effects. These strategies aim to create nanocarriers that maintain the stealth property during circulation (passive targeting) and then transform to a more cell-interactive form (active targeting) upon arrival at the target tumor sites (Figure 2). These nanocarriers will be referred to as "activatable" or "activated" nanocarriers hereafter.

These new targeting strategies explore the unique tumoral extracellular environment as a means to trigger such transformation. Tumors develop unique microenvironments such as slightly acidic pH[78] and a high level of proteinases.[79] The tumor extracellular pH is generally more acidic (pH 6.5 to 7.2[78]), due to the increased glycolysis and plasma membrane proton-pump activity of tumor cells, which make them produce more lactic acid than normal cells and leach out the acid to the extracellular milieu.[80,81] The overproduction of enzymes such as the matrix metalloproteinases (MMPs) is also common in most tumors, because MMPs are important for angiogenesis, metastasis, and other extracellular signaling events involved in tumor propagation.[79] MMP overexpression has been explored as a way of "turning on" imaging agents and locating tumors or other lesions.[82−86] The subsequent section will discuss nanocarriers that can be activated by pH or enzyme activity specific to the tumoral extracellular environment based on recently reported examples. Some of the pH-activated systems are also discussed in a recent review by Bae et al.[87]

pH-Activated Systems. A pH-responsive micelle system was developed using a poly(lactic acid)-*b*-PEG-*b*-poly(L-histidine) (PLA-b-PEG-b-polyHis) triblock copolymer.[37] This polymer has a pK_a of 7.0 and forms a "flowerlike" micelle (∼80 nm) with the PEG "petal" and the core consisting of PLA and polyHis (entrapping a hydrophobic drug) in water of pH 8. Below pH 7, the polyHis part is protonated, allowing the core to swell and release the drug.[37] Theoretically this system is supposed to remain a stable micelle that carries a drug in the core during circulation and then release the drug at once when it reaches the weakly acidic extracellular matrix of the tumors.[37] One potential limitation of this system is that the drug will enter the tumor cells as a free form (not as an encapsulated form); thus, it may be of limited use for treating the multidrug resistant tumor cells, which will efflux the free drug efficiently. This limitation is addressed by introducing a fragment of transactivating transcriptional activator protein (TAT peptide) in the system,[88] which is discussed later in this section.

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Another example of pH-responsive system is a micelle based on methyl ether poly(ethyleneglycol)-poly(β-amino ester) block copolymer, in which the methyl ether poly(ethyleneglycol) (MPEG) part forms a hydrophilic shell and the poly(β-amino ester) a hydrophobic core.[89] Poly(β-amino ester) has a pK_a of ∼6.5 and allows formation of micelles (40−60 nm) at pH >6.9.[89] At pH 6.4 the micelles released >71% of the drug in 6 h, whereas those at pH 7.4 did not release more than 20% in 24 h.[89] The *in vivo* study in B16F10 tumor-bearing mice showed that the micelles delivering doxorubicin effectively suppressed tumor growth and prolonged the survival as compared to free doxorubicin.[89] However, whether the pH-responsiveness contributed to the effectiveness (i.e., whether the antitumor efficacy is attributed to the passive targeting effect or the "activated" targeting effect) is not known, because it was not compared with a pH-insensitive system.

A nanocarrier system based on glycol chitosan[90] may be considered as an example of the activatable nanocarriers. A hydrophobically modified glycol chitosan (HGC) could encapsulate hydrophobic drugs such as camptothecin (CPT) by forming nanosized self-aggregates (280−330 nm) in aqueous media.[90] Significant tumor accumulation of HGC was observed as compared to those in the liver, lung, kidney, spleen, and heart tissue, with whole-body near-infrared (NIR) fluorescence imaging: approximately 2 to 3 times more nanocarriers accumulated at the tumor site than at the other organs.[90] Moreover, the CPT-HGC nanoparticles exhibited significant antitumor effects and high tumor targeting ability in nude mice bearing MDA-MB231 human breast cancer xenografts. The high tumor accumulation and antitumor effect were attributed to the stealth function and efficient cellular uptake of the HGC particles. While the authors did not explicitly mention, the significant tumor accumulation[90] and cellular uptake[91] may be explained by the chitosan's

pK$_a$ of 6.5.[92] Due to the pK_a, the HGC particles can remain un-ionized at pH 7.4 (serving as a stealth polymer) and protonate at the slightly acidic tumoral extracellular matrix (enhancing cellular uptake of the particles).

A liposome system reported by Sawant et al.[93] utilizes a pH-sensitive linker to achieve pH-responsive transformation from the stealth liposome to a cell-interactive form. This system includes a PEG layer attached to the liposome surface via a hydrazone linker, which cleaves at pH 5−6. The liposome surface is also conjugated to TAT peptide, which is shielded by the PEG layer at pH 7.4. At pH 5−6, the PEG layer detaches revealing the TAT peptide as the hydrazone linker hydrolyzes. A preliminary incubation of the pH-sensitive liposomes at pH 5 allowed the liposomes to enter the cells efficiently, whereas the pH-insensitive liposomes were much less efficient.[93] The limitation of this study is that the pH-responsiveness is shown at pH 5, which may be much lower than the pH of the tumoral extracellular matrix. However, the proof of principle is applicable for developing a new nanocarrier system that can be activated at a more realistic pH range.

A similar strategy is described in a study reporting a TAT peptide-based micelle system.[94] A polymeric micelle with TAT peptide termini (TAT micelle) is first created using a block copolymer of poly(L-lactic acid) (PLLA) and polyethylene glycol (PEG) (PLLA-*b*-PEG) conjugated to the TAT peptide.[94] The TAT micelle is then mixed with a diblock copolymer of poly(methacryloyl sulfadimethoxine) (PSD) and PEG (PSD-*b*-PEG), which adds an extra PEG layer on the micelle via the electrostatic interaction between TAT peptide (cationic) and PSD (anionic at pH >7.0). At pH 6.6, the PSD part turns to the un-ionized form. As a result, the micelle loses the PSD-*b*-PEG layer and reveals the TAT peptide termini (returns to the TAT micelle), which enhances the cellular uptake of the micelle. This micelle system is potentially capable of retaining the PEG layer during circulation and revealing the TAT peptide in a tumor-specific manner. A variant of this system employs poly(L-cystine bisamide-*g*-sulfadiazine) (PCBS) instead of PSD, in an attempt to overcome the nonbiodegradability issue of PSD.[95]

The aforementioned flowerlike micelle[37] was used in conjunction with biotin[96] or TAT peptide[88] to facilitate

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cellular uptake of nanocarriers upon extracellular activation. The micelle is made of a blend of two block copolymers: polyHis*-*b*-PEG and PLA-*b*-PEG-*b*-polyHis**-biotin[96] (or PLA-*b*-PEG-*b*-polyHis**-TAT[88]). Here, the micelle core consists of polyHis* and PLA from each polymer, and the shell is PEG, either in the form of straight chain or petal. At pH 7.4, the polyHis** of the PLA-*b*-PEG-*b*-polyHis**-biotin (or TAT) is un-ionized and stays associated with the polyHis*/PLA core, pulling the biotin (or TAT) termini close to the core and hiding them from the surface. Below pH 7.2, ionization of the polyHis** causes the PEG petal to expand, exposing the biotin (or TAT) on the micelle surface. This transformation enhanced cellular uptake of the micelles specifically at the pH lower than 7.2. Further pH decrease in the endosomes caused dissociation of micelles and intracellular release of encapsulated drugs.[96] Notably, the pH-activated TAT-micelle carrying doxorubicin was effective in killing both drug-sensitive and resistant cells. Antitumor activity and tumor accumulation of the pH-activated TAT-micelle were superior to those of a control micelle, which exposes TAT on the surface at all pHs.[88]

Enzymatically Activated Systems. Using enzyme over-production is another way of activating the nanocarriers in a tumor-specific manner. A drug−polymer conjugate was created by conjugating methotrexate to dextran via a peptide linker that could be cleaved by MMP-2 and MMP-9.[97−99] The biodistribution study shows that the drug−polymer conjugate achieves a tumor-targeting effect via the EPR effect.[98] The tumor distribution of the drug−polymer conjugate with a MMP-sensitive linker was not significantly different from that of an MMP-insensitive drug−polymer conjugate, suggesting that the MMP did not play a major role in tumor-targeted drug delivery. A potential reason would be that the endocytic uptake of drug−dextran conjugate occurred *before* the linker was cleaved by the enzyme.[98] It remains to be seen whether the enzymatic cleavage would occur prior to the uptake in much larger systems like nanocarriers as well, which may not enter the cells as fast as drug−dextran conjugates. Both MMP-sensitive and MMP-insensitive conjugates showed superior antitumor efficacy over free drug in the tumor models overexpressing MMPs.[99] On the other hand, the MMP-insensitive conjugate had severe systemic toxicity due to the unanticipated susceptibility of the peptide linker at the normal tissues.[99]

A liposomal gene carrier reported by Hatakeyama et al. also employs an MMP-sensitive peptide linker.[57] The MMP-sensitive peptide was used to link PEG to 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE).[57] The ternary conjugate of PEG−peptide−phospholipid formed a PEGylated liposome, from which the PEG was removed upon exposure to MMPs. The MMP-sensitive liposomes showed significantly higher gene transfection efficiency than MMP-insensitive liposomes in *in vitro* cell models overexpressing MMPs. In an *in vivo* study, the MMP-sensitive liposomes achieved prolonged circulation, resulting in 9−15 times higher AUC values due to the PEG on the surface than that of non-PEGylated liposomes. When MMP-sensitive and MMP-insensitive liposomes with equivalent PEG were compared for the *in vivo* gene expression per liposomes accumulated in the MMP-overexpressing tumors, the MMP-sensitive ones showed three times higher gene transfection efficiency than the MMP-insensitive ones. This result indicates that the MMPs overexpressed in the tumor extracellular matrix successfully removed the PEG from the liposomes and facilitated cellular uptake of the liposomes.[57]

Concluding Remarks

Extensive efforts to improve the effectiveness and safety of chemotherapy have brought about several tumor-targeted drug-delivery strategies. In particular, the leaky vasculature and impaired lymphatic system at the tumor tissues provided a unique opportunity to achieve tumor-selective distribution of nanocarriers. Moreover, the targeting molecules, such as antibodies, small molecular weight ligands, or aptamers, attached to the surface of nanocarriers contributed to drug delivery to the tumors by enabling the nanocarriers to more actively bind to specific tumor cells after extravasation. However, the *stealth effect*, which is necessary for selective tumor distribution of nanocarriers, and the *targeting effect*, which is needed for efficient entry to the target cells after tumor distribution, are hard to achieve simultaneously, often requiring a laborious effort to find the narrow window of "maximally targeted and maximally stealth" formulations.

Although in the early stage, recent efforts to create new nanocarriers that retain the stealth effect during circulation and transform to a cell-interactive form at the tumor site show good potential to achieve this goal. These strategies employ the unique tumoral extracellular environment, such as weakly acidic pH or overexpressed proteinases, as a molecular cue to activate the nanocarriers. For effective translation of *in vitro* proof of concept to *in vivo* efficacy, the following aspects should be considered. First, the activatable nanocarriers should have an optimal stability profile. Tumor-specific transformation of nanocarriers often results in destabilization of the nanocarriers (e.g., micelles, liposomes), which leads to burst-release of the drug at the extracellular matrix. These systems would have limited utility in overcoming multidrug resistance, for which cellular uptake of the nanocarriers and

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formation of an intracellular drug reservoir would be highly desirable. The destabilization of nanocarriers may be related to the inherent instability of the self-assembled systems, which has been recently observed by *in vivo* Förster resonance energy transfer imaging.[100] In this regard, polymeric nanoparticles may be considered as an alternative vehicle. On the other hand, if the nanoparticle is too stable, it may be limited in releasing sufficient drug inside the cells. Ideally, an activatable nanocarrier should remain stable during circulation and extracellular activation; once taken up by the cells, it should readily release the drug according to the spatiotemporal needs. To this end, intracellularly degradable polymers, which have gained increasing interest in nonviral gene delivery,[101−106] may be taken into consideration in designing new nanocarriers. Second, the transition from the stealth nanocarriers to the cell-interactive forms needs to be tumor-specific and sensitive to the stimuli so

that the carriers may not prematurely interact with normal cells and/or release drugs outside the target tissues. Further understanding of unique physicochemical and biological features of the tumor extracellular environment and development of stimuli-responsive materials would provide new opportunities for more efficient tumor-targeted nanocarriers.

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