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Expert Review

Poly(ethylene glycol)-modified Nanocarriers for Tumor-targeted and Intracellular Delivery

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Abstract. The success of anti-cancer therapies largely depends on the ability of the therapeutics to reach their designated cellular and intracellular target sites, while minimizing accumulation and action at non-specific sites. Surface modification of nanoparticulate carriers with poly(ethylene glycol) (PEG)/poly(ethylene oxide) (PEO) has emerged as a strategy to enhance solubility of hydrophobic drugs, prolong circulation time, minimize non-specific uptake, and allow for specific tumor-targeting through the enhanced permeability and retention effect. Furthermore, PEG/PEO modification has emerged as a platform for incorporation of active targeting ligands, thereby providing the drug and gene carriers with specific tumor-targeting properties through a flexible tether. This review focuses on the recent developments surrounding such PEG/PEO-surface modification of polymeric nanocarriers to promote tumor-targeting capabilities, thereby enhancing efficacy of anti-cancer therapeutic strategies.

KEY WORDS: intracellular delivery; long-circulation; poly(ethylene glycol); polymeric nanocarriers; tumor targeting.

INTRODUCTION

Cancer is the second leading cause of morbidity and mortality in the United States and the incidences are expected to continue on an upward trend for at least the near future. For instance, in 2007, approximately ten million cases of cancer will occur globally, with a total of around 1.5 million new cancer cases and over 560,000 deaths expected in the United States (1, 2). Nevertheless, over the past few decades significant advances have been made in fundamental cancer biology, allowing for remarkable advances in diagnosis and therapy of cancer. Strikingly though, the clinical translation of these advances lags far behind. A major hurdle is the successful delivery of novel therapeutic agents to the target site, while avoiding adverse damage resulting from systemic administration. While systemic drug delivery already hinges largely on physicochemical properties of the drug, such as size, diffusivity, and plasma protein binding affinity, tumors possess a dense, heterogeneous vasculature, and an outward net convective flow that act as additional hurdles to efficient drug deposition at the target site (3). Spatial release of potent, and often toxic, anti-cancer drugs at the target site can increase target efficacy and decrease non-specific damage.

To overcome some of these problems, key advances in tumor-targeted delivery have emerged. A significant advancement was made with the observation by Matsumura and Maeda (4) that tumors possess a unique physiology of fenestrated vasculature and poor lymphatic drainage, a characteristic that is now widely known as the *enhanced permeability and retention* (EPR) effect. By this mechanism, large gaps between adjacent endothelial cells in tumor neovasculature allows for passive targeting to the tumor site, while poor lymphatic drainage leads to enhanced retention of macromolecular therapeutics within the tumor mass, despite the presence of an outward net convective flow (5). Among the many tumor-targeting strategies that quickly emerged to increase site-specific localization of therapeutics by these means, the use of nanoparticle-based drug delivery systems has taken a predominant role in tumor targeting.

Despite this advantageous effect, a drug delivery device must be present in the circulation for long enough time to reach its intended target tissue. Plasma proteins, known as opsonins, can bind circulating drug delivery devices, including nanocarriers, and remove them from the circulation within seconds to minutes through the reticulo-endothelial system (RES) (6). Imparting a stealth-shielding on the surface of these drug delivery systems prevents opsonins from recognizing these particles, thereby limiting phagocytosis by the RES cells and increasing the systemic circulation time from minutes to hours or even days (6). Poly(ethylene glycol) (PEG) (known as poly(ethylene oxide) (PEO) when the molecular weight is greater than 20 kDa) modification has emerged as a common strategy to ensure such stealth-shielding and long-circulation of therapeutics or delivery devices. PEG-modification is often referred to as PEGylation, a term that implies the covalent binding or non-covalent entrapment or adsorption of PEG onto an object.

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Among the different delivery systems, nano-sized drug carriers are receiving considerable attention. The first generation of clinically approved nanotechnology-based drug delivery systems for cancer therapy used liposomes, as seen with liposomal daunorubicin (DaunoXome®) and doxorubicin (Doxil®), although development moved quickly towards other nanoparticle platforms, resulting in the further approval of nanocarrier based therapies, for example, Abraxane®, a formulation consisting of paclitaxel encapsulated into albumin nanoparticles. Through judicious selection of candidate therapeutics and appropriate functionalization of the nanocarrier systems, it is possible to develop fairly sophisticated multifunctional systems that can provide optimized anticancer therapy, a function that imparts particular use in intracellular delivery and sub-cellular localization of drugs. Since many chemotherapeutic drugs, and particularly gene therapeutics, would benefit from intracellular targeting, nanocarrier systems can be designed for non-specific or receptor-mediated cell uptake, intracellular drug protection, and intracellular target delivery (7).

Polymeric nanoparticles offer significant advantages over other nanocarrier platforms primarily since a tremendous versatility in polymer matrices allows for tailoring of the nanoparticle properties to meet the specific intended need. Other advantages of polymeric nanoparticles include ease of production, ease of surface modification, encapsulation efficiency of the payload, payload protection, large area-to-volume, slow or fast polymer degradation and stimuli-responsive polymer erosion for temporal control over the release of drugs, and feasibility of scale-up and manufacturing under Current Good Manufacturing Practices (cGMP) guidelines (8). Some examples of the most commonly used polymers for nanocarriers include, but by far are not limited to, synthetic polymers such as poly(D,L-lactide-co-glycolide) (PLGA), poly (L-lactic acid) (PLL), poly(epsilon-caprolactone) (PCL), polyalkylcyanoacrylates, and natural polymers such as gelatin, chitosan, and hyaluronic acid (9, 10).

This expert review will specifically focus on the recent developments in the use of poly(ethylene glycol) (PEG)/poly(ethylene oxide) (PEO)-modified long-circulation polymeric nanocarriers for tumor-targeting by passive and/or by active means. Additionally, novel evidence supporting the role of PEGylation in intracellular drug stability and/or delivery of anticancer drugs, gene-therapies, and RNA-interference (RNAi) therapies will be reviewed.

PEG SURFACE MODIFICATION AND LONG-CIRCULATION PROPERTIES

Among several strategies to impart particles with stealth-shielding, including surface modification with polysaccharides, poly(acrylamide), and poly(vinyl alcohol), surface modification with PEG and PEG co-polymers proved to be most effective, fueling its wide-spread use (6, 11, 12). PEG has a general structure of $HO–(CH_2CH_2O)_n–CH_2CH_2–OH$, encompassing a polyether backbone that is chemically inert, with terminal hydroxyl groups that can be activated for conjugation to different types of polymers and drugs. Amphiphilic block co-polymers, such as poloxamers and poloxamines, consisting of blocks of hydrophilic PEG (or PEO) and hydrophobic poly(propylene oxide) (PPO) are additional forms of PEG derivatives, often employed for modification by surface adsorption or entrapment (6, 12). The PPO, as the hydrophobic block, anchors onto or entraps within the surface of hydrophobic nanoparticle matrices. The poloxamers, commercially available as Pluronics® from BASF Corporation, are a-b-a type triblock copolymers (PEO–PPO–PEO) and poloxamines (or Tetronics®) are tetrablock copolymers of PEO–PPO joined by an ethylenediamine bridge ($(PEO–PPO)_2–x–(PPO–PEO)_2$) (13–15). The hydrophilic-lipophilic balance of these copolymers can be tuned through variations in the molecular weight of the "a" and "b" blocks. For example, Pluronic® F-108 NF (poloxamer 338) has a bulkier central block as well as longer side arms (a=122; b=56) as compared to Pluronic® F-68 NF (poloxamer 188, a=76; b=30). These differences can impart a significant change in the physicochemical properties of the triblock copolymer that in turn influences its applicability. For example, surface modification with poloxamers of varying a:b ratios, therein, influenced biodistribution of PCL nanoparticles in that 74% of Pluronic® F-68 NF modified nanoparticles accumulated within the liver 1 h after injection, while only 67% of Pluronic® F-108 NF modified nanoparticles accumulated in the liver, still significantly better than liver accumulation of unmodified nanoparticles (83%), supporting the observation that PEG surface-shielding helps avoid recognition by the RES system (16).

Surface modification of the polymeric nanoparticles can be achieved through covalent means, by grafting of PEG chains onto the nanoparticle surface, and similarly through the use of co-polymers, whereby PEG is covalently attached to another polymer type. However, PEG modification can also occur by non-covalent means of surface adsorption or entrapment into the nanoparticle matrix (6). A handful of examples regarding PEG modification by covalent and non-covalent means are presented in Table I. PEG offers the advantage that it is non-toxic and non-immunogenic, leading to approval by the United States Food and Drug Administration (FDA) for internal use in humans and inclusion in the list of inactive ingredients for oral and parenteral applications (17). PEG is available in a wide range of molecular weights up to several million daltons, which influences its elimination from the body, since it is not biodegradable. Yamaoka *et al*. have shown that PEG with a molecular weight of up to 20 kDa is primarily excreted through the renal system, however, PEG chains with a higher molecular weight transition from urinary to fecal excretion (18).

In addition to the variations in molecular weight, determined by the number of repeating units, PEG chains can be synthesized in two conformations, as either linear or branched (19). The protective (stealth) action of PEG is mainly due to the formation of a dense, hydrophilic cloud of long flexible chains on the surface of the colloidal particle that reduces the hydrophobic interactions with the RES. The tethered and/or chemically anchored PEG chains can undergo spatial conformations, thus preventing the opsonization of particles by the macrophages of the RES, which leads to preferential accumulation in the liver and spleen. PEG surface modification, therefore, enhances the circulation time of molecules and colloidal particles in the blood (11, 20–22).

The mechanism of steric hindrance by the PEG modified surface has been thoroughly examined. (12). The water

Table I. Illustrative Examples of Poly(ethylene glycol)-modified Nanocarriers

Complex	Application	Outcome	References
Non-covalent PEG-modified Nanocarriers			
PEG-block-poly (epsilon-caprolactone)	Tuning the hydrophilicity	Hybrid gold nanoparticles were synthesized by RAFT polymerization	[68]
Multi-arm PEG-poly(L-lactide)	Biodegradable hydrogels	Hydrogels with high storage moduli	[69]
Poly(N-vinylpyridine)-graft-PEG copolymer	Protein repellent and stable interface	Semiconductor material for self-assembling into micelle	[70]
PEG-block-poly(epsilon caprolactone)	Nanoencapsulation of lipophilic prodrugs	Geldanamycin analogue against breast cancer	[71]
PEG-DSPE	Encapsulation of octathyprophine	Solubility and stability enhancement	[72]
PEG-peptide-DOPE conjugate	Tumor specific cleavable lipid	*In vivo* cancer gene therapy	[73]
Covalent PEG-modified Nanocarriers			
PEG-modified cationic gelatin	Non-viral gene carrier system	Gene transfection and expression was found to be significantly increased	[74]
TAT-derived peptide covalently coupled to PEG-PEI	Nanocarrier for DNA delivery	Non-viral gene carrier for lung therapy	[60]
PEG-poly(D,L-lactide)	Tissue adhesive hydrogel	Tissue adhesive applications	[75]
Fluroalkyl double-ended PEG	Chlorambucil-Tempol adduct	High chlorambucil entrapment efficacy	[76]
PEG-poly(L-lactic acid) amphiphilic di-block co-polymers	Thermo-stimuli	Self-association and micelle formation	[77]
PEG- poly(D,L-lactic acid)-PEG	Dexamethasone nano-aggregates	Baloon-catheter treatment	[78]
Folate-PEG-polyaspartate hydrazone doxorubicin)	Doxorubicin encapsulated in polymeric micelles	pH-triggered drug release	[79]

PEG=Poly(ethylene glycol)

molecules form a structured shell through hydrogen bonding to the ether oxygen molecules of PEG. The tightly bound water forms a hydrated film around the particle and repels the protein interactions (23). In addition, PEG surface modification may also increase the hydrodynamic size of the particle decreasing its clearance, a process that is dependent on the molecular size as well as particle volume (24). Ultimately, this aides in greatly increasing circulation half-life of the particles (6, 21, 25). The technology of PEG-modification is readily in use, and a few examples of the many PEG-modified nanocarrier products developed for tumor targeting are presented in Table II.

The size, molecular weight, and shape of the PEG fraction and the linkage used to connect it to the entity of interest determine the consequences of PEGylation in relation to protein adsorption and pharmacokinetic properties (like volume of distribution, circulation half-life, and renal clearance). When formulated into colloidal particles, the PEG density on the colloidal surface can be changed by adjusting the molecular weight (chain length) of PEG and molar ratio (grafting efficiency) of PEG incorporation. Longer PEG chains offer greater steric influence around the colloidal entity, a similar phenomenon seen when the grafting density is increased, regardless of PEG chain length. Longer PEG chains may also collapse onto the nanoparticle surface, thereby also providing a hydrophilic shield (26). As discussed previously, steric-shielding enhances circulation time of therapeutics, thus it is not surprising that colloidal particles modified with 6.5 mol% PEG generally have a longer circulation time ($t_{1/2}$ of 170 min) than particles modified with 2.5 mol% PEG ($t_{1/2}$ of 80 min). Interestingly, branched derivatives of PEG generally have an increased half-life over unbranched PEG chains, however at 7 or greater mol% surface modification, both the branched and unbranched derivatives show a similar steric effect.

Many reports exist to support the pharmacokinetic improvements seen with use of PEG-modified nanocarriers over un-modified nanocarrriers. For example, PEG modification of gelatin nanoparticles increased circulating half-life from 3 to 15 h over unmodified gelatin nanoparticles, accompanied by a three-fold decrease in total body clearance (27). This prolonged circulation hereby significantly increased tumor retention from a half-life of 29 to 38 h, an improvement that can result in a significant enhancement of therapeutic efficacy. Similarly, PEG-modification of PLGA nanocarriers improved circulation time, whereby only 5% of unmodified particles remained in the circulation within 5 min of administration, but as much as 25% of PEG (molecular weight of 5,000 Da) remained circulating (22). Interestingly, PLGA particles that had been modified with 20,000 Da molecular weight PEG retained up to 50% in the circulation within the first 5 min, an observation that supports the increased stealth properties and decreased clearance of longer PEG chains.

Table II. Illustrative Examples of Comparative Pharmacokinetics Between the Conventional and PEG-modified Nanocarriers

Delivery System	Application	Outcome	References
Long-circulating PEG-modified gelatin nanoparticles	Intracellular delivery	Enhanced cytotoxicity and prolonged circulation time	[30]
Long-circulating PEG-modified gelatin nanoparticles	Lewis lung carcinoma	2-fold higher concentration in blood-pool and longer residence due to steric repulsion	[29]
Paclitaxel-PEG-nanoparticles	Tumor-targeted drug delivery	Complete tumor regression and accumulation of paclitaxel within solid tumor mass	[28]
Doxorubicin-PEG-PHDCA nanospheres	Brain Tumor-targeted drug delivery	Maximum tolerated dose increased by 1.5-fold and reduced toxicity	[35]
PEG-coated gadolinium nanoparticles	Tumor site-specific targeting	Significant accumulation in the tumor mass and cellular internalization	[80]
PEG-modification and multi-mineralization of scFv	Cancer therapy	Longer serum half-life, 14.5-fold higher accumulation in tumor cells	[81]
PEG-PEI-siRNA nanoparticles	Tumor tissue selective delivery	Protein expression within tumor and tumor regression	[43]
Antiestrogen-PEG-coated nanospheres	Breast cancer treatment	Decrease in tumor size	[16]
TAT–PEG–PEI conjugated nanocarriers	Lung cancer	Significantly higher transfection efficiency	[60]
TNF-alpha loaded Stealth x nanoparticles	Tumor targeting	Extended plasma half life and 2.85-fold increase in AUC	[37]
Doxorubicin-PEG 400 conjugated nanoparticles	Tumor targeting	Comparative biodistribution revealed highest concentration in tumor cells	[82]
Methotrexate-PEG 4,000 conjugate lipid nanoparticles	Cancer treatment	Prolonged circulation time and can be useful for systemic and controlled targeting of drugs to tumor cells.	[83]

PEG=Poly(ethylene glycol)

ILLUSTRATIVE EXAMPLES OF TUMOR-TARGETED DELIVERY

The tumor targeting and prolonged circulation properties of PEG modified nanocarriers, as discussed previously, ultimately allows for significantly elevated drug concentrations at the tumor site (28, 29). Prolonged circulation increases the probability that the nanocarriers reach the tumor interstitium, where, mediated by the EPR effect, it is possible to accumulate a larger fraction of the administered dose than the tumor would see when drugs are administered parentally without a carrier. Moreover, PEG surface modification is conducive to the incorporation of active targeting ligands, mediated by the ease with which PEG can become functionalized. Together, these properties of PEG modulation allow for the development of efficient anti-tumor therapeutic strategies.

Passive Targeting with PEG-modified Nanocarriers

Over the last several years, our group has developed a number of PEG/PEO modified nanocarrier systems for tumor-targeted drug and gene delivery (16, 30–34). PEG-modified PCL nanoparticles were developed for systemic delivery of tamoxifen in breast cancer. In this study, it was observed that PEG modification not only reduced particle size and aggregation, but significantly enhanced the circulation time allowing for approximately 18% of the injected dose to accumulate in the tumor mass within 1 h, in an *in-vivo* model of human breast adenocarcinoma, a feat that is in stark contrast to the 5% tumor accumulation of the nanocarriers lacking PEG (16). In a study carried out by Brigger *et al.*, PEG-coated poly(hexadecylcyanoacrylate) nanospheres loaded with doxorubicin were found to increase the maximum tolerated dose 1.5-fold higher compared to conventional nanoparticles, hence reducing toxicity (35). Xu *et al.*, administred paclitaxel via PEG-modified polycyanoacrylate nanoparticles, and observed a 4.8-fold higher accumulation of paclitaxel in the solid tumor mass coupled with significant tumor regression with time (36). Likewise, surface modification with methoxyPEG onto cyanoacrylate particles (PEG-PHDCA) carrying a recombinant-TNF-alpha therapeutic load, resulted in decreased macrophage phagocytosis and decreased opsonization, giving rise to an increase in circulating half-life of TNF-alpha from 28.2 min to 11.33 h (37). PEG surface modification thereby resulted in a 2.85-fold greater TNF-alpha peak concentration and 7.44-fold AUC at the tumor site, a phenomenon that interestingly increased as PEG molecular weight increased, an result that the authors attribute to an increase in thickness of the soluble surface layer coupled with a decrease in distance between neighboring PEG chains (37). Kaul and Amiji have developed long-circulating PEG-modified type B gelatin nanoparticles for tumor-targeted gene delivery, that even *in-vitro* increased transfection efficiency from 43 to 61% when the gelatin nanocarriers were PEGylated (38), suggesting that PEG-surface modification enhanced cell uptake of the nanocarriers, or prevented damage of the labile DNA-load in the endosomal pathway. As conventional with PEG-surface modification, the plasma half-lives, mean residence times,

and area-under-the-curve of PEG-modified nanoparticles were significantly higher as compared to the unmodified gelatin nanoparticles, when this study was translated *in-vivo* in a mouse model of Lewis Lung carcinoma. It is not surprising then that the PEG-nanoparticles had an increased residence half-life in the tumor (121 h) over plain gelatin nanoparticles (19 h), supporting the three-fold increase in reporter gene expression in the tumor cells (39).

Active Targeting with PEG-modified Nanocarriers

While it has been demonstrated that PEG surface modification of nanocarriers causes a greater accumulation of drug at the tumor-site by passive targeting, active targeting of the carrier can aide in selection of the target cell-type within the tumor site and internalization of the nanoparticles to a greater extent inside the target cells. A wide variety of tumor targeting ligands exist all coupled to nanocarriers through PEG-linkage. These approaches include small molecule ligands such as folate (40, 41) and thiamine (42), peptides such as RGD (43), and sequences identified by phage display (44, 45), proteins such as transferrin (46), lectins (47), antibodies and antibody fragments (48, 49), polysaccharides such as galactose (50), and aptamers (51). Regardless of the targeting moiety, the principle outcome is essentially the same, mainly improved tumor cell recognition, improved tumor cell uptake, and reduced recognition at non-specific sites. PEG surface modification provides an advantage whereby the terminal groups of PEG can be functionalized to reactive groups for covalent coupling. Most commonly, PEG is functionalized to reactive carboxylic acids, amine, or sulfhydryl groups, allowing for efficient covalent attachment of the wide assortment of targeting ligands by amide bonding or disulfide bridge formation, as illustrated in Fig. 1. Hereby, PEG remains as a surface modification, while also functioning as a linker for covalent attachment of active targeting moieties. Folate targeting is a widely used active targeting ligand since the receptor recognizing the vitamin folic acid is commonly over-expressed on a wide variety of tumor types (52). Folate coupling to the surface of PLGA nanoparticles via PEG, resulted in a greater intracellular nanoparticle uptake of the nanocarriers in folate receptor (FR) positive KB tumor cells over uptake of the nanocarriers lacking the targeting ligand, as well as over uptake of the carriers to FR negative A549 tumor cells (41). Interestingly, a study that targeted gadolinium-containing nanoparticles to FR positive KB nasopharyngeal carcinoma, where folic acid was covalently coupled to the PEG surface coating, revealed that active targeting did not improve the ability of the nanocarrier to reach the tumor site over PEG modification alone from the systemic circulation, but that active targeting did greatly enhance the ability of the carrier to internalize into the tumor cells (53). Similarly, folate targeting of DNA-loaded nanoplexes, where folate was also covalently coupled to the PEG-shield, resulted in an increased amount of cell-adhesion and intracellular gene expression compared to the folate lacking PEG-nanoplexes, where the carriers were targeted to not only FR-positive KB cells but also to prostate specific membrane antigen (PSMA) positive LNCaP cells (54). By the same means, the tumor targeting moiety transferrin has also been coupled to PEG to actively target long-circulating nanoparticles, causing them to accumulate to a greater fraction in transferrin-receptor positive tumor masses, while avoiding adhesion of the particles to TR-negative cells (55).

Besides facilitating active targeting by means of incorporating receptor ligands, peptides, aptamers, and even macromolecules such as proteins (including antibodies) can be coupled to PEG by similar covalent bonds, hereby illustrating the great versatility of PEG in active targeting of nanocarriers in addition to its passive targeting properties. The peptide RGD preferentially binds to particular integrins which are often over-expressed on the surface of endothelial cells of tumor vasculature. Covalently attached RGD to PEG, functionalized to bear a reactive amine group, by an amide bond, was incorporated covalently to poly(ethyleneimine) and nanoplexed with siRNA as an anti-cancer therapeutic strategy (43). In this case again, active targeting enhanced intracellular uptake of the siRNA therapeutic load to both HUVEC (human endothelial) and N2A (murine neuroblastoma) cells, although tumor-site accumulation was essentially the same for the PEG-modified nanoplexes as it was for the RGD-PEG-modified nanoplexes (43). Aptamers are DNA or RNA based oligonucleotide strands which,

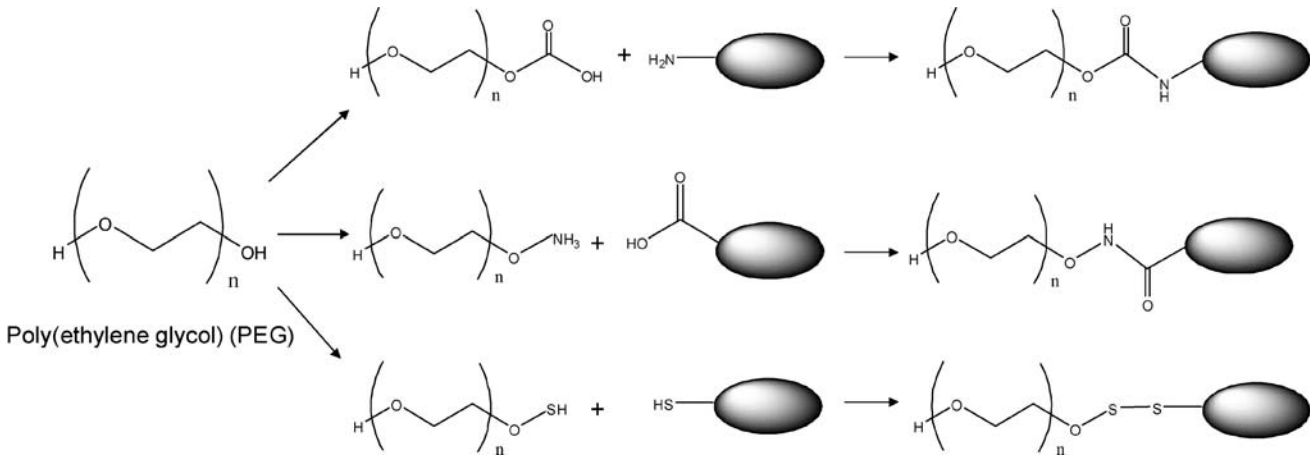

Fig. 1. Representative chemical schemes for covalent attachment of active targeting ligands such as proteins, peptides, aptamers, and small molecules to poly(ethylene glycol).

through their unique tertiary folding pattern, can recognize antigens with high affinity and specificity. Tumor cell targeting by incorporating RNA-aptamers against PSMA, through amide bonding of the targeting moiety to the PEG shield to docetaxel carrying PLGA-PEG nanoparticles, again resulted in at least 20% more cell kill of LNCaP prostate cancer cells *in-vitro* coupled with significant shrinkage of the tumor *in-vivo* (51). As previously seen with other active targeting moieties, PEG-nanoparticle docetaxel treatment was significantly more efficacious than untargeted docetaxel, while the actively targeted aptamer-PEG-nanoparticle treatment further improved overall outcome of the therapy (51).

While PEG surface modification of nanoparticles targets the therapeutic load to the tumor site far more efficiently than unmodified nanoparticles, it appears that active targeting of the nanocarrier aids in cell-specific recognition and internalization of the therapeutic carrier. PEG shielding in addition to active targeting not only allows the particle to retain its stealth properties and accumulate at the tumor site by passive targeting, but the PEG shield provides an assembly for versatile incorporation of various active targeting ligands, thereby greatly improving the anti-cancer efficacy of the therapeutic strategy.

One major limitation of PEG surface modification is the possibility for reduced specificity with the tumor cells. PEG surface modification may mask targeting ligands or surface functional groups thereby incorporating the possibility of reduced association or interaction with the cells. This might also lead to decreases in the intracellular delivery efficiency of the modified nanoparticles.

ILLUSTRATIVE EXAMPLES OF INTRACELLULAR DELIVERY

Tumor directed therapies experience hurdles not only in their ability to reach the tumor site, but also in their ability to localize within the tumor cells and reach their intended intracellular target, all the while avoiding degradation in the endosome-lysosome (56). Intracellular localization to target organelles is particularly of importance, for example, in gene therapeutics and chemotherapeutics that target DNA and RNA production, such as fluorouracil, gemcitabine, and doxorubicin, whose localization outside the nucleus results in therapeutic failure (56). Additionally, internalization of therapeutics into the endosomal-lysosomal pathway can lead to degradation of sensitive bio-therapeutics or ionization of weakly acidic/basic chemotherapeutics resulting in organelle entrapment. An illustration of intracellular targets and targeting hurdles is presented in Fig. 2. Regardless of whether the therapeutic agent localizes efficiently within the tumor mass, a failure of the therapeutic to reach its intended intracellular target prevents a response.

Delivery to Intracellular Targets

Intracellular target localization is crucial for the action of both conventional chemotherapeutic drugs and novel bio-therapeutics such as gene and protein therapies in the treatment of cancer. While topoisomerase inhibitor drugs, for example, act in the nucleus, taxanes act in the cytosol, and proapoptotic drugs often act in the vicinity of the mitochondria (56, 57). Similarly, gene therapeutics must be directed towards the nucleus, with the exception of siRNA treatments which function in the cytosol. Failure of therapeutics to reach intracellular targets, or subjection of therapeutics to lysosomal degradation, is a frequent reason for poor bioavailability and rejection of promising therapeutic candidates.

Strategies have emerged to overcome the limitations of intracellular delivery, resulting in improved therapeutic efficacy of anticancer therapies. To aid in intracellular internalization of nanocarriers, cell penetrating peptides, such as the trans-activating transcriptional activator peptide (TAT) have been incorporated on the nanoparticle surface (58). TAT peptides are arginine-rich sequences derived from the viral coat of HIV-1 that aide in crossing cell membranes due to its strong cell surface adherence, and translocating to the nucleus, independent of receptors (59). Like other active targeting agents, TAT can be covalently coupled to the tip of functionalized PEG chains. Such incorporation of the TAT peptide on the surface of PEG-shielded PEI-DNA nanoplexes, for example, led to a six-fold higher target transfection efficiency *in-vivo* (60). Interestingly, this enhanced therapeutic efficiency of the TAT-PEG-nanoplex was six-fold over transfection efficiency of the TAT-PEI particles lacking PEG, suggesting that the PEG-shielding remains necessary for site-specific localization (60). Interestingly, it has recently been suggested that TAT can enhance intracellular localization of therapeutics to the mitochondria (61), a property that is suggested to occur from electrostatic attraction between the positively-charged cell penetrating peptides and the highly negatively-charged mitochondria. Nevertheless, this tool provides a useful platform to target therapeutics to the mitochondria where particular gene- and protein-therapeutics and pro-apoptotic drugs find their site of action.

Besides active targeting to enhance intracellular and organellar delivery, formulation of pH- responsive entities into the polymeric nanocarriers also appears to enhance intracellular localization. Oishi *et al*., (62) utilized this principle to develop polymeric micelles for gene delivery, whereby a pH-responsive poly(silamine) (PSAO) portion was included in the PEG-based tri-block polymer that composed the micelles. It was found that these particles significantly enhanced transfection efficiency, as demonstrated by the transcription of a reporter gene, to Huh-7 (hepatocarcinoma) cells compared with the PEG micelles that lacked the pH-responsive block, a phenomenon that the authors attribute to an endosomal disruptive function of the PSAO portion, whereby the gene-therapy load can escape degradation and/or sequestration (62). It is commonly known that sequestration of protons by weakly basic chemicals, termed the "proton-sponge effect," can facilitate such endosomal disruption, thereby releasing the therapeutic load into the cytoplasm, an issue that is particularly of importance in saving easily degradable gene therapeutics (63). This principle of endosomal disruption was illustrated with similar inclusion of positive charges to polymeric micelles, in the development of PEG-PE micelles containing lipofectin lipids (LL), to enhance the chemotherapeutic potential of the drug paclitaxel (64). Microscopy revealed that the PEG-PE-LL formulation facilitated intracellular uptake and caused disruption of endosomal structure with consequent release of the drug load into the cytosol, a phenomenon that was not

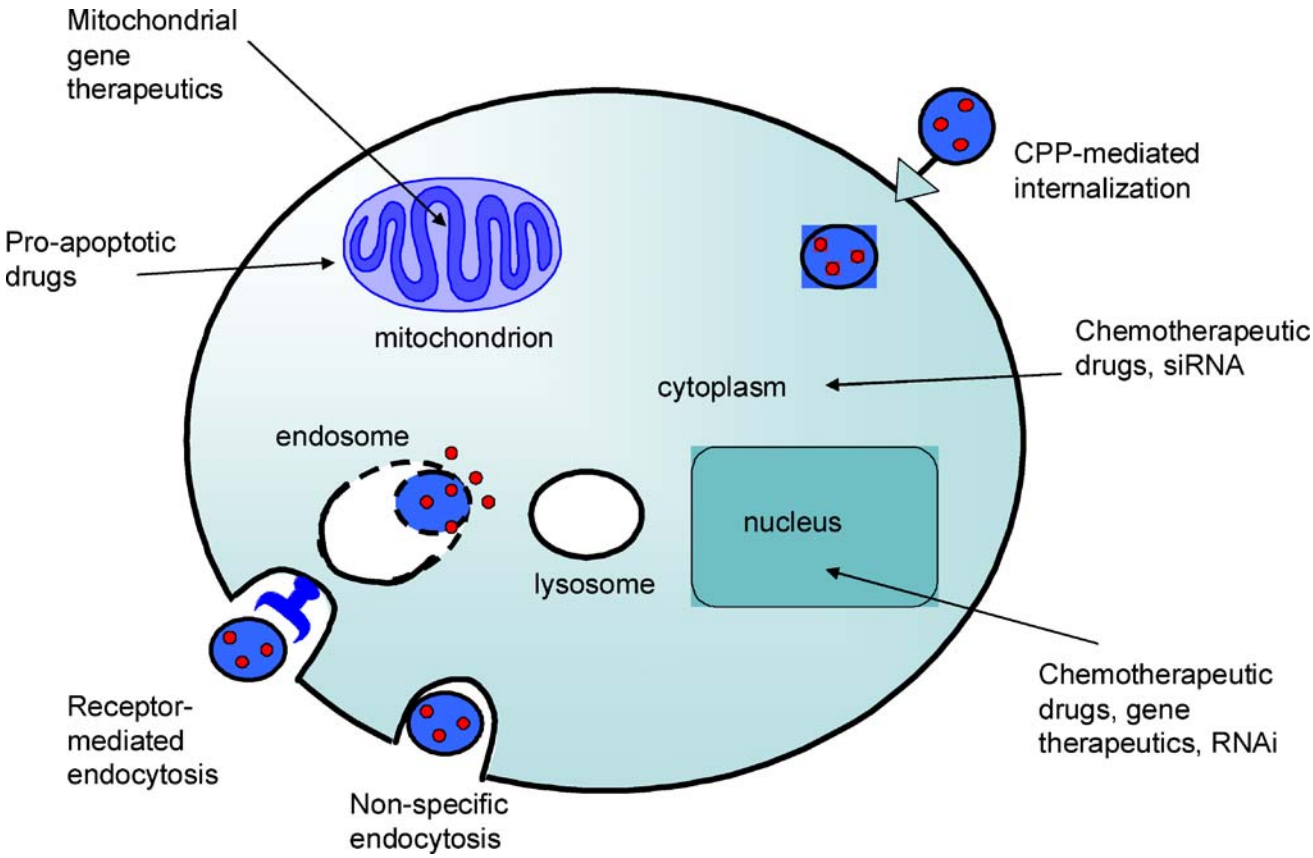

Fig. 2. Schematic illustration of the basic intracellular targets including cytosol, nucleus, and mitochondria for drug and gene delivery and the various barriers/mechanisms to targeting of anticancer therapeutics (adapted from Torchilin (7)).

observed with the PEG-PE micelles lacking the pH-responsive component. The overall outcome was that therapeutic efficacy of this formulation was far greater in both A2780 and BT-20 cancer cells, compared to the plain PEG-PE micelles (64).

Overall, such results demonstrate that proper intracellular targeting greatly improves therapeutic efficacy, a feat to which PEG-modified polymeric nanocarriers can play a substantial role.

Intracellular Stability and Target Localization

Therapeutic agents meet many hurdles in the body that must be overcome for their action to be successfully completed. Besides the hurdles of target-specific localization both to the tissue mass and to intracellular targets, stability of the therapeutic and the carrier are as important to ensure that the maximum dose reaches the intended target site. PEG shielding can help promote this stability of the therapeutics. PEG shielding of DNA loaded nanoplexes protected the DNA load from degradation in the presence of up to 2.5 U/μg DNaseI, while the DNA load of the un-shielded nanoplexes was degraded in the presence of a mere 0.1 U/μg DNaseI (60). In the same set of experiments, PEG shielding of the PEI nanoplex (where PEI is known to have compensated biocompatibility) proved significantly less cytotoxic to A549 (lung epithelial) cells with two-thirds less pro-inflammatory TNFα production than from the unshielded PEI nanoplexes (60). Similarly, Mao, *et al.* showed that PEG surface-shielding of siRNA bearing poly(ethyleneimine) (PEI) polyplexes generally protected the siRNA load from RNAse degradation, but that the protection increased with an increase of PEG chain length. In this case, 20 kDa PEG chains provided the most protection, where >80% of the siRNA load remained intact even in the presence of 15 mIU RNAse A/μg siRNA (26). Not surprising then, that PEG-protection of these polyplexes resulted a roughly 50% greater degree of gene knockdown than un-PEGylated PEI polyplexes *in-vitro* (26).

The non-adhesive nature of PEG as a surface coating has been reported in many cases to prevent nanoparticle aggregation, a problem that severely compromises nanoparticle use. For example, PEG surface modification prevented the aggregation of both PEI- and cyclodextrin-based nanoparticles, a problem that did occur with the un-PEG-modified PEI and cyclodextrin particles (65). Similarly, PEG modification not only prevented aggregation of surface modified liposomes, but also significantly delayed content leakage of the therapeutic load from the particles as compared to un-modified nanoparticles; a phenomenon that was dependent on PEG chain length and increasing percent PEG incorporation into the particle (66).

CONCLUSION AND FUTURE OUTLOOK

PEG/PEO surface modification is a favored component of drug delivery systems. PEG has been employed as a stealth shield to promote prolonged circulation by protecting

the therapeutic load and the carrier from uptake by the RES, metabolism and excretion. Additionally, PEG/PEO modification has proven as a useful scaffold to incorporate active targeting ligands and influence intracellular target localization, further enhancing therapeutic success. Through PEG modification, nanocarriers demonstrating both active and passive targeting properties can be developed for cancer therapy, thereby greatly enhancing therapeutic success through preferential location of the drugs at the tumor site coupled with enhanced intracellular uptake and intracellular targeting. Furthermore, PEG-shielding also seems to encourage particle stability on the shelf and *in-vivo* and reduce toxicity of the polymeric nanoparticle complexes.

With the aide of PEG, engineering of drug delivery systems is moving in the direction of "smart" devices, drug delivery systems that are to specifically homed to and responsive only in their target environments. Such engineering has been recently illustrated in the work by Sawant *et al.* (67) who developed PEG-PE based micelles that have alternating low molecular weight PEG surface-strands, to which the cell penetrating peptide TAT is coupled, and high molecular weight PEG strands attached to the particle by pH-labile bonds, to which the tumor recognition antibody 2G4 is coupled. The result is that the high molecular weight PEG chains shied the TAT-peptide attached to the low molecular weight PEG chains, preventing non-specific internalization of the particle mediated by the eager TAT sequence. Following prolonged circulation and uptake into the tumor mass mediated by both the EPR effect and the active targeting agent on the surface of the carrier, the pH-responsive bonds are cleaved in the acidic environment of the tumor, thereby releasing the high molecular weight PEG strands from the carrier and exposing the TAT-peptide for enhanced intracellular uptake and intracellular target localization, specifically located to the target tumor cells. Such engineering strategies allow, through the use of PEG, for the development of delivery vehicles that are long-circulating, non-immunogenic, targeted, and environment responsive, thereby not only greatly increasing the potential for therapeutic success, but also limiting interactions of the therapeutics at non-specific sites.

The role of PEG has proven crucial in the evolution of drug delivery systems, especially for tumor targeting and treatment. The versatility that has been demonstrated with PEG will allow for the exploration of novel uses and continuous improvements of anti-cancer treatments.

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