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Peptide mediated cancer targeting of nanoconjugates

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Abstract

Targeted use of nanoparticles *in vitro*, in cells and *in vivo* requires nanoparticle surface functionalization. Moieties that can be used for such a purpose include small molecules as well as polymers made of different biological and organic materials. Short amino acid polymers--peptides can often rival target binding avidity of much larger molecules. At the same time, peptides are smaller than most nanoparticles and thus allow for multiple nanoparticle modifications and creation of pluripotent nanoparticles. Most nanoparticles provide multiple binding sites for different cargo and targeting peptides which can be used for development of novel approaches for cancer targeting, diagnostics and therapy. In this review, we will focus on peptides which have been used for preparation of different nanoparticles designed for cancer research.

Introduction

Recent biomedical interest in nanoparticles has spurred on many studies focusing on nanoparticle uptake. Frequently, nanoparticle characteristics in and of themselves have profound influence on cell-nanoparticle interactions. For example, electropositive gold nanoparticles were recently found to depolarize cell membranes and modulate calcium release in cells *in vitro* (1), while *in vivo* studies found that positively charged nanoparticles fall prey to neutrophil extracellular traps more easily than any other nanoparticles (2). However, while “core” nanoparticle characteristics should be taken into consideration in any study, in this review, in the interest of brevity, we will focus only on moieties that can be used to target nanoparticles into different cell types.

Cell type and tissue differences lie at the core of any biomedical research or therapeutic approach. Innate differences between cancer and healthy cells are at the crux of any cancer therapy or diagnostic approach. Unique features of sick cells are the target for any type of medical intervention. In some cases, innate behaviors of cells can be harnessed for therapy —such as use of radioactive iodine to treat thyroid cancers. More often, researchers have used antibodies that recognize cell surface specific epitopes. For example, in nuclear medicine, radioactive atoms have been conjugated to antibodies targeting cancer cells. More recently, antibodies have been used to target nanoparticles to specific cancer types. For example, an antibody against epidermal growth factor receptor (EGFR)—cetuximab, has been used to target gold nanoparticles into pancreatic cancer cell lines with variable EGFR expression, both *in vitro* and in xenograft mouse models (3). Nevertheless, while antibodies are an interesting and viable option for nanoparticle targeting, they are relatively large: with their approximate size of 10×15nm they match the size of many nanoparticles. In order to realize full pluripotentiality of nanoparticles it is desirable to attach multiple molecules onto each nanoparticle. This in turn dictates that nanoparticle ligands be small in size and peptides fit that requirement excellently. Moreover, different peptides can be used not only for targeting cells based on their surface epitopes, but also to penetrate cells or tissues, cause cell toxicity etc. Targeting nanoparticles to tumor support structures such as neovasculature or stroma has also gained interest over the past few years.

Use of peptides for delivery of different materials into cells has a relatively recent history. Among the first such molecules was the Tat peptide, which was derived from the trans activating transcriptional activator of Human Immunodeficiency Virus 1. Due to its ability to enter cells, this type of peptide was named “cell penetrating peptide” (CPP). While many more “advanced” CPP peptides have been developed subsequently, Tat is still in use and provides a reliable vehicle for delivery of different cargo into cells.

In addition to CPPs, many peptides used for building of nanoconjugates provide cell or tissue specific targeting regardless of, and even in absence of intracellular delivery. These peptides can be considered to be “pre-designed” as they were developed to target specific target epitopes, as an alternative to antibodies. Most often these polymers are designed to mimic natural ligands for different cellular proteins, embedded in cell membrane or present inside cells. Less frequently, they are peptide sequences derived from antibodies. A special subcategory of targeting peptides are those that are “modified” through interaction with their target: e.g. proteolytic degradation by a specifically localized enzyme can provide the way for enzymatically triggered, cell-type specific cargo delivery. Another category of targeting peptides include those that are derived from random peptide libraries selected through reiterative target panning. Most frequently peptide libraries are developed in conjunction with phage displays. The random peptides in such case are presented on the surfaces of phage envelopes, and high titers of different phage particles are exposed to a more or less complex assembly of different epitopes at the same time. Through a reiterative selection process specific phage clones are detected, each carrying peptides targeting a particular epitope that is often identified only subsequently. Phage display has successfully provided a rapid approach for random synthesis and re-iterative selection of peptides for binding specific targets. However, selection of desirable peptides for any particular purpose is still a challenge. It is likely that new developments in nanoparticle research may provide alternative ways to increase the speed of discovery of peptides of biomedical significance.

In general, peptide (conjugated) nanoparticles employ different peptides in several ways:

- to target nanoparticles which carry drugs or diagnostic agents into specific cancer cells. Peptides used can be
  - based on ligands associated with specific cells/organs
    - tumor cells themselves
    - tumor support structures such as stroma or neovasculature
  - based on random peptides libraries, and selected based on interaction with specific epitopes
- to target the nanoparticles, once inside cells, into specific subcellular compartments

Moreover, other than as targeting agents, peptides have also been used to build the nanoparticles. This has been done so far in two types of situations:

- when peptides which can be viewed as therapeutic agents on their own have been conjugated to nanoparticles—i.e. peptide confers cytotoxicity
- when peptides are structural building blocks of the nanoparticles themselves.

These last two types of nanoparticle associated peptides will be touched upon only briefly in this review, as both represent well developed research fields in their own rite, with numerous review articles dedicated to each.

1) Nanoparticles with “pre-targeted” peptides

1.a) Peptides serving as ligands for known tumor epitopes

1.a.1) Peptides for targeting of nanoparticles to tumor cells: One of the most significant difficulties with any type of cancer therapeutic agent is the fact that treatments often bind to some of the normal tissues and organs as well or perhaps even better than they are able to target the tumor tissue itself. This is may also be a problem with diagnostic imaging agents as well. Cancer targeting nanoparticles are not an exception to this rule, and nanoparticle uptake by the reticuloendothelial system (RES) is one of the persistent problems in bio-nanotechnology. As with proteins or liposomes, conjugating poly(ethylene glycol) (PEG) to nanoparticles facilitates their resistance to RES uptake. PEG is a non-toxic, non-immunogenic linear or branched polyether terminated with hydroxyl groups. PEGylation, covalent conjugation of PEG to different molecules and molecular structures, changes the physical and chemical properties of the molecules bound to PEG, their conformation, electrostatic binding, and hydrophobicity. PEGylation therefore generally increases the solubility and stability of the molecules to which it is bound (especially by reducing proteolysis), decreases immunogenicity, modulates their retention in blood, and affects renal excretion (4). PEGylation of nanoparticles has been successfully used to modulate uptake. For example, 20–50 nm PEGylated AuNPs much reduced uptake by the RES compared to non-PEGylated counterparts (5). Similarly, the biodistribution of targeted and PEGylated single wall carbon nanotubes (SWNTs) in mice has also shown improvements over the distribution of targeted SWNTs without PEG (6).

Despite the possibility of evading the RES, a good therapeutic index for any treatment, including nanoparticulates requires specific targeting and rapid uptake of the by the target tissue leading to an increased differential uptake of the nanoparticles at the desired sites compared to non-targeted tissues. Examples of targeted nanoparticles are numerous; however, in many cases the same or similar peptides are employed conjugated to different types of particles. For that reason in this review we will focus in the first place on the peptides and the organs/tissues or cancer types that have been targeted.

Bombesin (BBN) peptide (QQRLGNQWAVGHLM) (Table 1) and its analogs can be used to target gastrin-releasing peptide (GRP) receptors; *in vivo* GRP receptors are overexpressed in glioblastomas, small cell lung, gastric, pancreatic, prostate, breast, cervical, and colon cancers. Recently, 16nm gold nanoparticles functionalized by a high load of thioctic acid–bombesin peptide were used to target prostate tumor xenografts in SCID mice (7). Using normal and prostate tumor bearing mice they showed that AuNP-BBN exhibit high binding affinity to the tumor and confirmed that AuNP-BBN constructs are GRP receptor specific and accumulate with high selectivity in GRP receptor rich pancreatic acine in normal mice and also in prostate xenografts in immunodeficient mice.

Somatostatin (AGCKNFFWKTFTSC) and its analogs can be used to target somatostatin receptors overexpressed in both small cell and non-small cell lung cancers. For example, one such analog is peptide P2045 which has been used to deliver radionuclides for diagnosis and therapy in lung cancer patients (8). Use of somatostatin analogs for in vivo nanoparticle therapies still awaits development. However, an analog from Drosophila, allatostatin 1 (APSGAQRLTYGFGL-NH2), has been used for cellular targeting of quantum dots to mammalian cells carrying somatostatin or galanin receptors (9). Since specific subtypes of galanin receptors can be found in head and neck squamous cell carcinoma it may be possible to adapt this peptide for targeting nanoparticles to each or both of these two cancers.

Follicle-stimulating hormone analogs such as FSH β chain carrying peptide FSH-33 (YTRDLVYKDPARPKIQKTCTF). FSH-33 is a peptide that can be used to target FSH

receptors in ovarian cancer. Nanoparticles of 51 to 78nm were prepared from maleimide - poly(ethylene glycol)-poly(lactic acid) (maleimide PEG-PLA) or methoxy poly(ethylene glycol)-poly(lactic acid) (mPEG-PLA), empty or carrying paclitaxel. When FSH-33 was used to target paclitaxel-filled nanoparticles, the best therapeutic outcomes were obtained in mice carrying human ovarian cancer Caov-3 xenografts. This study measures at what concentration different nanoconstructs and drugs inhibit the growth of xenograft tumors by 50% (the so called half maximal inhibitory concentration or IC50). The presence of paclitaxel decreased the IC50 of the nanoparticles 4 fold, and the presence of FSH-33 as well increased it by an additional 2.7 fold (10).

LyP-1 peptide (CGNKRTRGC) has high specific binding for cells carrying p32 protein on cell surface, such as MDA-MB-435 melanoma cancer cells. This peptide sequence carries thiol and amine groups which made it possible to use “click” chemistry to conjugate them to azide carrying superparamagnetic nanoparticles. Von Maltzahn and others used assembled nanoparticles to target MDA-MB-435 xenograft tumors (11). This work showed that, for uptake by MDA-MB-435 cells, LyP-1 nanoconjugates far outperformed control nanoconjugates carrying control peptide (without NKRTR motif) or non-conjugated azide nanoparticles. In xenograft models, “click” nanoparticles were able to stably withstand the systemic circulation for hours (>5 h circulation time) following intravenous administration. These nanoconjugates have also accumulated in tumors and penetrated interstitial cells expressing p32. In a different study, in conjunction with quantum dots, this peptide was found to recognize lymphatic vessels (12).

Fibroblast growth factor analogs can be used to target cells expressing fibroblast growth factor receptors (FGFRs). This receptor family is often expressed both on tumor cells and neovasculature. Truncated human basic fibroblast growth factor peptide (tbFGF) was recently used to achieve targeting of liposomes carrying chemotherapeutic drugs (13). This long peptide contains both the bFGF receptor binding site and a part of the heparin-binding site, which allows it to bind FGFRs on cell surface, without stimulating cellular proliferation. The amino acid sequence of this peptide is KRLYCKNGGF FLRIHPDGRV DGVREKSDPH IKLQLQAEER GVVSIKGVCA NRYLAMKEDG RLLASKCVTD ECFFFERLES NNYNTY.

Liver cancer targeting peptide FQHPSFI was used to deliver liposomes carrying therapeutic DNA into HepG2 hepatocarcinoma cells in culture. This peptide showed differential accumulation in hepatocarcinoma cells (HepG2) compared to normal liver cells (THLE-3), embryonic kidney cells (AD293) or breast cancer cells (MCF-7) (14).

Peptide GFE (CGFECVRQCP ERC) **and peptide F3** (KDEPQRRSAR LSAKPAPPKP EPKPKKAPAK K) were found to target endothelial cells of lung blood vessels and tumor vasculature, respectively, in *in vivo* experiments with quantum dots (15). The previously mentioned peptide LyP-1 (CGNKRTRGC) was also tested in the same study. Following intravenous delivery quantum dots conjugated to these three peptides accumulated in lung tissue (GFE), blood vessels (F3) or lymphatic vessels (LyP1). According to previous research it was expected that GFE should bind to the endothelial cells in lung blood vessels (16), F3 to blood vessels and tumor cells in various tumors (17) and LyP-1 to lymphatic vessels and tumor cells of certain tumors (12).

Peptides targeting epidermal growth factor receptor (EGFR) can be expected to be very useful for the treatment of large number of tumors, as this receptor is upregulated in a large number of different cancers and almost all squamous carcinomas. In one of the recent examples of work with the carbon based nanoparticulates, single wall carbon nanotubes were loaded with cisplatin, labeled by quantum dot conjugation and targeted to head and

neck squamous carcinoma cells through the conjugation of the complete EGF (18). Testing in a mouse xenograft model demonstrated a significant tumor volume decrease in the test cohort compared to mice treated with SWNT-cisplatin control. In a different study, gold nanoparticles carrying a cargo of gemcitabine as an anticancer drug were targeted to EGFR pancreatic cancer cell lines in xenograft mice. Targeting moiety in this case was anti EGFR antibody cetuximab (3).

Tripeptide RGD and numerous alternative peptides containing this sequence target integrin αvβ3 on endothelial cells very well, therefore RGD sequences are most often used for neovasculature delivery of cargo, including many different types of nanoparticles. While that topic will be given more attention in one of the later sections, it is worth noting at this time that RGD can also be used to target αvβ6 integrin which is overexpressed in head and neck cancers (19). In a recent example, RGD peptide has been used to target gold nanoparticles into oral cancer cells *in vitro* (20).

Peptides targeting neuropilin-1 receptor (CendR) developed by the Ruoslahti laboratory, containing sequence R/KXXR/K, promulgate extravasation, tissue penetration, and cell entry of attached cargo. These peptides have the property of universal tissue penetration if this amino acid motif is exposed at the C terminus (21). This property can be used to make the peptide targeting be dependent on local enzymatic activity of the target tumor. For example, a hybrid peptide can be synthesized to consist of tumor-homing peptide covalently bound to the penetrating peptide in such a way as to shield its C terminus. When the target tumor tissue expresses a protease capable of proteolytically processing the hybrid peptide and releasing the CendR, a very fine-tuned cargo delivery can be accomplished using this approach. A RGD-CendR hybrid peptide, iRGD (CRGDK/RGPD/EC), exemplifies the capabilities of these peptides (22). This hybrid peptide was conjugated to the 130nm a nanoparticle made of albumin-embedded paclitaxel. This nanocomplex binds to integrins in the neovasculature in tumors developed in mice both in orthotopic human breast tumor (BT474) and human prostate tumor (22Rv1) models. Proteases present on site cleave iRGD into a peptide CRGDK/R which penetrates into tumor tissue and delivers 10 times more nanoparticle cargo into a tumor than a conventional nontargeted nanoconjugate (22).

In short, many different peptides based on pre-existing targeted molecules with identified targets have been used to target nanoparticles to cancer tissues (Table 1). In addition to the peptides currently in use, there are many more that have not yet been used in conjunction with nanoparticles. However, these peptides have been employed for targeting other types of cargo for diagnostic or therapeutic agents. A few examples of these potential new targeting agents are provided below.

Alpha melanocyte stimulating hormone or **α-MSH peptide** and its analogs can be used to target α-MSH receptors frequently present on metastatic melanoma cells (23). Wild-type alpha-MSH peptide is responsible for the regulation of skin pigmentation. A much shorter peptide (called CCMSH) was found useful for single photon emission computed tomography (SPECT). Peptide (Acetyl-CCEHdFRWCKPV-NH2), capable of housing radionuclide Technetium-99m, was found to be a capable tumor targeting agent for diagnostic imaging (24). So far, this peptide has not been used for nanoparticle targeting.

Antitumor-antibody-derived peptides based on the sequence EPPT were investigated for breast cancer uptake with cell lines MCF-7, MDA-MB-231 and T47-D, where they internalized well (25). Since the presumed target for these peptides is mucin 1, and because this protein is also over-expressed in ovarian, prostate and colon cancer, it is possible that it may find uses in targeting nanoparticles in any one of these cancers. Another transmembrane glycoprotein CD44 is also often associated with different cancers. Several

variants of this protein are associated with different metastatic diseases including breast and head and neck cancers. Interfering with binding between CD44 and its ligand hyaluronan (HA) is therefore another field of great interest for potential peptide use (26).

Enterotoxin (STh) from *Escherichia coli* is a 19 amino acid sequence (NSSNYCCELC CNPACTGCY) which binds to the guanylate cyclase C receptor. This receptor is present in high density on the apical surface of normal intestinal epithelial cells as well as on the surface of human colon cancer cells. At present, STh analogues are used to target radionuclides to human colon cancers (27).

1.a.2) Peptides for targeting of nanoparticles to tumor support structures: Tumor development not only on tumor cell proliferation but also on the presence of normal cells recruited by the tumor to provide tumor stroma and neovasculature. In some tumors, these "support structures" create formidable obstacles to delivery of chemotherapeutic drugs. For example, the desmoplastic reaction typical for pancreatic cancer is considered a major obstacle to the successful therapy of this disease. In addition, in all solid tumors neovasculature development is an essential step in cancer progression. For these reasons, there is a great interest in providing new modalities for cancer treatment that compromise the integrity of tumor support structures. Examples of peptide targeted nanoconjugates in this section are also listed in Table 1.

A cyclic decapeptide CGLIIQKNEC preferentially binds to the fibronectin-fibrin complexes in the extracellular matrix of different tumors. Using click chemistry this peptide was associated with dendrimers previously carrying gadolinium for magnetic resonance imaging, resulting in a nanoglobular contrast agent which was tested in mice carrying MDA MB-231 breast tumor xenografts (28).

Peptide WIFPWIQL is a recently identified amino acid sequence that can be used for targeting neo-angiogenesis. It targets a novel endothelial cell membrane protein BiP/GRP78 the function of which is upregulated by vascular endothelial growth factor. WIFPWIQL targeted 100 nm DSPEPEG liposomes (prepared from Distearoyl phosphatidylcholine, distearoyl phosphatidylglycerol, cholesterol and distearoyl phosphatidylethanolamine-conjugated to PEG 2000) carrying doxorubicin caused a significant reduction of the neovasculature in a dorsal air sac mouse tumor model (29).

Tripeptide RGD and numerous variant peptides containing this sequence have been used to target integrin $\alpha v\beta 3$ which is expressed exclusively on endothelial cells of the neovasulature and whose with specific ligands play a key role in angiogenesis (30). In treatment of solid tumors in the recent years, therefore, angiogenic endothelial cells have been a major target (31). The tripeptide arginine–glycine–aspartic acid (RGD) has been used to target tumor endothelial cells with different drugs or nanoparticles and we will provide here only a very few examples.

In many cases, RGD targeting of nanoconstructs was used simply on its own. For example, RGD targeted and PEGylated single wall carbon nanotubes (SWNTs) have been used for xenograft targeting in mice (6). PLGA (a copolymer of lactide and glycolide)-based nanoparticles grafted with either RGD peptide or RGD-peptidomimetic agents (RGDp) and loaded with Paclitaxel were both successfully used to treat breast cancer xenograft tumors in mice (32). Similarly, RGD targeted, doxorubicin carrying nanoparticles made of distearoylphosphatidylcholine (DSPC), cholesterol, dioleoylphosphatidylethanolamine (DOPE), distearoylphosphatidylethanolamine (DSPE)-mPEG2000, have been used to target meatasteses (33).

More recently, RGD tripeptides have been used more and more often together with other peptides (20) or in the context of hybrid peptides and molecules. For example, the RGDyK peptide has been used for delivery of a prodrug whose activation depended on the presence of the bioreductive enzyme DT-diaphorase (34). In this case, neovasculature targeting in association with the enzymatic activity anticipated at the target site were used synergistically to maximize the therapeutic index of the drug (SN38). In a previous example (22) RGD was used both to target and to temporarily "inactivate" CendR peptide. Following an enzymatic reaction after the hybrid peptide nanoconjugates reach the neovasculature, RGD is removed and CendR delivers its cargo to the target tissue.

1.b) Peptides selected by "random" iterative procedure(s) and used in nanoparticles—Previous knowledge about the precise target on tumor cells is not always necessary when developing a peptide conjugated nanoconstruct. Often a phage display or similar iterative technique with a trial and error approach can be used to select peptides targeting specific cell/tissue types. In all such cases it is necessary to, eventually, identify which receptors really take the peptide(s) developed through the selection process. For example, polyamines are known to be taken up well by many different cell types; however, it was only recently shown that the membrane molecule responsible for their uptake by cells was identified as L-carnitine transporter hCT2 encoded by the human gene SLC22A16 (35).

One of the best and most potent iterative approaches for peptide selection is phage display. This is an in vitro selection method that allows reiterative selection of polypeptides with desired properties from large collections of peptide variants (36). These random peptides are displayed on capsid proteins of bacteriophages infecting *Escherichia coli*. Most commonly, filamentous phages such as f1, M13 and fd are used for this purpose. Nevertheless, complex capsid phages T4, T7, and λ can also be employed. Phage display has been used to investigate interactions between peptides and "simple targets" such as purified proteins and nucleic acids or more complex substrates such as whole cells.

For example, synthetic peptides from the cell membrane protein mucin 1 were used as relatively simple "bait" in a phage display (25). Panning revealed several phage peptides with the sequence EPPT which subsequently were found to bind with high affinities for MCF-7, MDA-MB-231 and T47-D breast cancer cell lines *in vitro*. Phage display "baits" often come from biomarker screening efforts. For example, a microarray research endeavor uncovered Hepsin, a type II transmembrane serine protease as a potential target in prostate cancer. Fortunately, the same protein shows low levels of expression in normal prostate. Through phage display work, peptides targeting this protein were found and subsequently tested conjugated to fluorescent nanoparticles. Peptide loaded particles were used for selective targeting of mouse xenografts with (LNCaP) and without (PC3) hepsin expression (37). The same group developed peptides targeting plectin 1 which is overexpressed in pancreatic ductal adenocarcinoma (PDAC) (38). These targeting peptides were used conjugated to fluorescently labeled superparamagnetic nanoparticles (so called Cross Linked Iron Oxide or CLIO) and tested in a mouse PDAC model by magnetic resonance imaging. The same imaging technique was used in a transgenic mouse breast cancer model to test superparamagnetic, amino dextran-coated iron oxide (SPIO) nanoparticles conjugated to the peptide CREKA discovered by phage display. Previously, this peptide was noted as absent in normal tissues but abundant in tumor stroma (39).

Lately phage panning with different cells and complex substrate assemblies is becoming more common. So called landscape panning with PC3 prostate cancer cells as the desired target and the liver cell line HEK293 as the negative target lead to the discovery of a peptide (DTDSHVNL) with high specificity and selectivity for prostate targeting (40). Interestingly, peptides which affect cell behavior can also be selected by phage display. For example,

peptides that can detect new substrates on irradiated neovasculature (41) or a series of peptides (CTGKSC, PAVLG and LRVG) enabling transcytosis across enterocytes and follicle associated epithelium cells could also be identified by phage display (42). It is likely that many peptides from phage display library searches may be successful in targeting nanoparticles to specific cancers while avoiding healthy cells.

In its turn, nanotechnology can aid in the utilization of peptides. For example, “free” peptides often show binding that is much weaker to the substrate than that to the complete proteins or peptides displayed on bacteriophages. Helms and others (43) have used such peptides conjugated to a dendrimer and showed an enhanced affinity of a polyvalent collagen binding peptide-dendrimer as compared to native collagen binding protein. In another example, a library of peptides bound to fluorescently labeled CLIO nanoparticles (38 nm mean diameter) was delivered to a series of cell lines. These cells were pancreatic cancer cells (PaCa-2), macrophage cell line (U937), resting primary human macrophages, activated primary human macrophages; and human umbilical vein endothelial cells (HUVEC). Uptake of 146 different nanoconjugates from the library was different in different cells, thus providing information about the best matches between different peptide-CLIOs and each tested cell type (44).

2) Peptides targeting nanoparticles into specific subcellular compartments

While nanoparticle associated cytotoxicity has often been found following treatment of cells with high concentrations of nanoparticles, specific sub-cellular locations may have different thresholds for the presence of nanoparticles. A recent example of this paradigm came from work with gold nanoparticles (20). Gold nanoparticles of 30nm diameter were used, tagged either with the integrin targeting RGD peptide alone, or with RGD and a cell penetrating peptide (KKKRK). In human oral squamous cell carcinoma, which overexpresses $\alpha v\beta 6$ integrins, (but not control cells) uptake of RGD & KKKRK gold particles into the cell nucleus resulted in cell mortality due to the interruption of the cell cycle. At the same time, cytoplasmic accumulation of nanoparticles bearing only RGD had no adverse effect on cell viability (20). This differential sensitivity of cells to the presence of nanoparticles in different cellular compartments may be developed into an additional vehicle for use of nanoparticles as anti-cancer agents.

Many peptides “extracted” from different viral proteins have been developed in recent years and their use for nanoparticle functionalization is frequent. They have shown variable success in crossing obstacles such as the cell membrane, endosomal membrane and nuclear membrane. Gold nanoparticles modified with the **nuclear localization signal (NLS)** from **the large T antigen of SV40** virus have been used to study nuclear transport (45). Sequence KKKRK was found to bind to importin-R, a nuclear transport protein leading to nanoconjugate entry to the nucleus through the nuclear pores. In a different study Tkachenko and others (46) compared the same NLS peptide sequence matching the **large T antigen of SV40** (CGGGPKKKRKVGG) with several other peptides: a NLS peptide derived from **HIV Tat protein** (CGGRKKRRQR RRAP), a NLS from **adenovirus fiber protein** (CGGFSTSLRA RKA) and finally a peptide containing an **integrin binding domain** (CKKKKKKGGR GDMFG). In three different cell lines (HeLa, HepG2, and 3T3/NIH), 20nm gold nanoparticles functionalized with bovine serum albumin conjugated with the different peptides localized into different subcellular compartments; sometimes nanoconjugates entered the nucleus sometimes they remained in the cytoplasm. The conclusion of this work was that different endocytosis processes were employed by different cell lines and for different nanoconjugate combinations.

In order to be able to follow the *in vivo* distribution of hematopoietic (CD34+) and neuronal (C17.2) progenitor cells Lewin and others (47) labeled these cells with nanomaterial that

would make them suitable for magnetic resonance imaging, fluorescent microscopy and chelation of radionuclides. This labeling was done by loading the cells with functionalized conjugates of dextran coated 5nm iron oxide CLIO nanoparticles. Into the dextran coating were added dextrans conjugated to a 14 amino acid peptide sequence CKYGRRRQRRKKRG containing the **Tat peptide** sequence GRKKRRQR and covalently linked to the FITC fluorophore. Nanoconjugate loaded cells could be followed in mice, and the labeled CD34+ cells could also be separated and further purified by magnetic separation after *in vivo* migration.

In addition to Tat based peptides, which were based on the discovery of endocytosis properties of Tat protein 20 years ago (48) other peptides are often used to fortify cellular uptake of nanoparticles. Some of these include the third helix of the homeodomain protein Antennapedia (49), a peptide originally derived from an anti-DNA monoclonal antibody (50), and a peptide derived from the VP22 protein from herpes virus (51)

Olson and others (52) developed activatable cell penetrating peptides molecules. These short polycations were attached to neutralizing polyanions, via protease-cleavable linkers and conjugated to dendrimeric nanoparticles. Moreover, these dendrimers were labeled with the fluorescent dye Cy5, magnetic resonance contrast agent gadolinium, or both and used in mice to detect residual tumor and metastases as small as 200 μm. Tumor uptake of dendrimeric nanoconjugates with activatable cell penetrating peptides was 4- to15-fold higher than for their peptide free dendritic counterparts.

3) Nanoparticles carrying peptides as therapeutic agents

Another category of peptide functionalized NPs includes those cases where the peptide used on its own is universally toxic, but attached to the NP it can be targeted and its toxicity harnessed. Cell killing peptides (Table 1) can sometimes be self-assembled into biodegradable nanoparticles or attached to non-peptide scaffolding. For example, a **cationic alpha-helical peptide based on the sequence KLAKLAK** is universally cytotoxic causing membrane disruption. Recently, a (KLAKLAK)2 peptide was integrated into a peptide amphiphile that self-assembles into cylindrical nanofibers (53) While in this circumstance these biodegradable nanoparticles were used to treat cultured breast cancer cells, it is anticipated that cytotoxic peptide(s) modified in this way may provide a safe agent for in vivo anti-cancer treatment.

Another universally cytotoxic peptide is **melittin**, a portion of the larger peptide contained in bee venom (54). This 26 amino acid α-helical peptide (GIGAVLKVLT TGLPALISWI KRKRQQ) causes cell death through cytolysis, and, in order to be useful *in vivo* must be targeted specifically to the tissue of interest. Targeting was achieved through incorporation of melittin into fluorocarbon nanostructures and targeted to cells with αvβ3 integrin expression through the presence of peptidomimetic vitronectin. Use of these nanoconstructs lead to a reduction of tumor volume in syngeneic B16F10 mouse melanoma tumors and human melanoma cells in culture (55).

While melittin and KLAKLAK peptides disturb the cell membrane, a group of tetrapeptides isolated from different Myxobacteria —**tubulysins** are toxic to cells through a different mechanism. Tubulysins cause depolymerization of cell microtubules, which prevents completion of mytosis and leads to apoptosis of proliferating cells. A thiol derivative of tubulysin A was covalently attached to a linear, h-cyclodextrin based polymer through a disulfide linker and used to treat different human cancer cell lines in culture and two nude mouse xenografts: with HT29 human colon or H460 non small cell lung carcinoma. In mice, tumor growth delay matched that which could be achieved by paclitaxel but without adverse effects such as loss of body weight (56).

4) Peptides as an integral component of the nanoparticle makeup

Nanoparticles made mostly or entirely of peptides are an attractive choice for nanotechnology because they can be prepared by self-assembly and are biodegradable. Self assembled peptide based nanoparticles can be created through the “covalent capture” mechanism, which is used naturally for assembly of fibrilar proteins such as collagen and elastin, or through use of peptide derivatives such as peptide amphiphiles or pi-stacking systems. Many laboratories are using different chemistries to create peptide based nanostructures *in vitro* (57, 58). Among these are, for example, self-assembling peptide amphiphiles which can be prepared either so as to have no particular biological effect on cells, or to carry bioactive peptide epitopes (59).

Similarly, when an amphipathic peptide linker is used as an integral part of the perfluorocarbon (PFC) nanoparticles it was possible to alter the nanoconstructs by post-conjugation of melittin (GIGAVLKVLTTGLPALISWI KRKRQQ) or “mutated” melittin peptides. This allowed the investigators to perform an extensive comparative study for different bioactive peptides in which the nanoparticle source was the same batch of PFC nanoparticles (60).

5) Considerations for work with peptides and peptide targeted nanoconjugates

In introduction we mentioned that any complete consideration of interactions between cells and nanoparticles must include investigation of the nanoparticle itself. Material that was used to create nanoparticle, size and surface charge of the nanoparticle, propensity to accumulate serum proteins on its surface, capacity to engage activity of immune system etc. all have to be studied for a complete knowledge of nanoparticles. Many excellent review articles have been published on this topic (61, 62,63). Due to the space constraints of this review, however, we will continue to focus only on peptides used in conjunction with nanoparticles.

Many different cancer targeting peptides were developed to allow for homing of diagnostic or therapeutic agents to specific tumors. In order to improve their specificity these targeting peptides are sometimes developed as “hybrid” molecules, targeting two epitopes at the same time. A careful choice of targets makes hybrid peptides more cell type specific, or may make the targeting into a two-step process, again increasing cell type specificity. The possibility of multiple peptide conjugations on the same nanoparticle makes peptide-nanoconjugates particularly likely to benefit from these options. Nevertheless, work with peptides can also lead to some potential problems that need to be considered.

Important considerations pertaining to peptide use include questions such as: (i) is the peptide-receptor binding resulting in peptide-nanoparticle internalization or not; (ii) is the peptide-receptor binding triggering a cell signaling cascade; (iii) is the final charge and molecular weight of the entire construct leading to accumulation of nanoparticles in normal tissues, etc. Sometimes, it is possible to draw hypothesis about such issues based on the work with other peptide targeted molecular composites such as PET contrast agents. For example, use of gastrin-releasing peptide (GRP) receptor agonist and antagonist: Demobesin 4 and Demobesin 1 (respectively) has shown that GRP receptor antagonists are better suited for tumor targeted PET imaging than agonists (64).

An example of issues associated with the final charge and molecular weight of the entire construct has been shown through the work of Borgman and others (65). The authors tested N-(2-hydroxypropyl) (HPMA) copolymer-RGDfK conjugates with different molecular weights. *In vitro* studies with endothelial cells showed that copolymer conjugates of approximately 43, 20 and 10 kD all actively bind to the $\alpha_v\beta_3$ integrin. However, biodistribution data have shown a very high accumulation of 43 kD conjugate in kidneys

(max 210% ID/g) and a proportionally much lower tumor accumulation (max 1.8% ID/g). The authors believed that increased negative charge content causes increased kidney accumulation with a loss of tumor accumulation in vivo.

Another example comes from the recent work by Chanda et al. (7) whose bombesin functionalized AuNP with a core size of ~16 nm and a hydrodynamic size of ~155 nm delivered IV accumulated equally well in the RES organs liver and spleen as in the GRP-receptor-rich pancreatic acine in normal mice. When IP delivery was done, accumulation occurred mostly in pancreas.

Conclusions

Peptide targeting of nanoparticle is steadily gaining ascendance in cancer research. Peptides created by phage display or other reiterative random library approaches are tested as targeting agents attached to nanoparticles even as their target epitopes are as yet in the process of discovery. Peptides derived from different larger proteins or ligands are rapidly being developed for use with nanoparticles as well. As the number of peptide-nanoconjugate examples increases new ideas on how to use such polyvalent assemblies are being formed. New ways to combine peptides with each other and with various diagnostic or therapeutic molecules have been made available by nanobiotechnology, and these novel agents often have very different properties than the starting materials used in preparation of the nanoconjugates. Peptide targeting itself is becoming combinatorial—the use of more than a single peptide, the use of peptides also serving as enzymatic substrates etc. all unite to provide completely novel approaches for cancer treatment by nanoconstructs.

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Table 1

Peptides used as components of nanoparticles.

Nanoparticles with peptides based on ligands for different cancer related targets				
Peptides serving as ligands for known tumor epitopes				
Peptides for targeting of nanoparticles to tumor cells				
Peptide/Protein	**Target**	**NP**	**Amino acid sequence**	**Reference**
Bombesin (BBN)	Gastrin-releasing peptide receptors	Au NPs, 16nm	QQRLGNQWAV GHLM	7
Allatostatin 1	Somatostatin receptors Galanin receptors	Q-dots	APSGAQRLTY GFGL	9
Follicle-stimulating hormone analog FSH-33	Follicle-stimulating hormone receptors	maleimide PEG-PLA; mPEG-PLA 51 to 78nm	YTRDLVYKDP ARPKIQKTCT F	10
LyP-1 peptide	p32 protein	SPIO	CGNKRTRGC	11
	Lymphatic vessels	Q-dots		12, 15
Fibroblast growth factor analogs	Fibroblast growth factor receptors	liposomes	KRLYCKNGGF FLRIHPDGRV DGVREKSDPH IKLQLQAEER GVVSIKGVCA NRYLAMKEDG RLLASKCVTD ECFFFERLES NNYNTY	13
Hepatocarcinoma targeting peptide	HepG2 liver cancer cells	liposomes	FQHPSFI	14
Peptide GFE	Endothelial cells of lung blood vessels	Q-dots	CGFECVRQCP ERC	15
EGF	Epidermal Growth factor receptors	SWNTs	complete EGF	18
cetuximab		Au NPs, 2nm	anti EGFR antibody	3
RGD peptide	Integrin αvβ6	Au NPs, 30nm	RGD alone or KKKRK alone	20
			RGD & KKKRK on same NP	
CendR	Neuropilin-1 receptor	Albumin-embedded Paclitaxel, 130nm	CRGD(K/R)GP(D/E)C	22
Peptides serving as ligands for known tumor epitopes				
Peptides for targeting of nanoparticles to tumor support structures				
Peptide/Protein	**Target**	**NP**	**Amino acid sequence**	**Reference**
Matrix targeting peptide	Fibronectin-fibrin complexes	Dendrimers	CGLIIQKNEC	28
Neo-vasculature targeting peptide	Endothelial cell membrane protein BiP/GRP78	liposomes, 100nm	WIFPWIQL	29
Neo-vasculature targeting RGD peptides and isomers	Integrin αvβ3	SWNTs	RGD	6
		PLGA NPs	RGD	32
		(DSPE)-mPEG2000	RGD	33
iRGD peptide (RGD-CendR hybrid peptide)	Integrin αvβ3 followed by Neuropilin-1receptor targeting	Albumin-embedded Paclitaxel, 130nm	CRGD(K/R)GP(D/E)C	22
Peptide F3	Tumor vasculature	Q-dots	KDEPQRRSAR LSAKPAPPKP EPKPKKAPAK K	15
LyP-1 peptide	Lymphatic vessels	Q-dots	CGNKRTRGC	12, 15
Stroma targeting	Tumor stroma	SPIO	CREKA	39
Peptides selected by “random” iterative procedure(s) and used in nanoparticles				
Peptide/Protein	**Target**	**NP**	**Amino acid sequence**	**Reference**
Prostate cancer targeting	Hepsin	CLIO, FITC, 38.7 nm	IPLVVPL	37
Pancreas cancer targeting	Plectin 1	CLIO, Cy5, 38.7 nm	KTLLPTP	38

Nanoparticles with peptides based on ligands for different cancer related targets				
Peptides serving as ligands for known tumor epitopes				
Peptides for targeting of nanoparticles to tumor cells				
Peptide/Protein	**Target**	**NP**	**Amino acid sequence**	**Reference**
Stroma targeting	Tumor stroma	SPIO	CREKA	39
Nanoparticles targeted by peptides into specific subcellular compartments				
Peptide/Protein	**Target**	**NP**	**Amino acid sequence**	**Reference**
SV40 large T NLS	cell nucleus via importin-α	Au NP, 20nm	KKKRK or CGGGPKKKRKVGG	45, 46
HIV Tat protein NLS	cell nucleus via importin-β	Au NP, 20nm	CGGRKKRRQR RRAP	46
		CLIO, FITC	CKYGRRRQRRKKRG	47
Adenovirus fiber protein NLS	cell nucleus and cytoplasm	Au NP, 20nm	CGGFSTSLRA RKA	46
Nanoparticles carrying peptides as therapeutic agents				
Peptide/Protein	**Target**	**NP**	**Amino acid sequence**	**Reference**
KLAK peptide	membrane disruption cytotoxicity	biodegradable peptide NPs	KLAKLAK(2)	53
Melittin	membrane disruption cytotoxicity	fluorocarbon NPs	GIGAVLKVLT TGLPALISWI KRKRQQ & RGD	55
Tubulysin A	Tubulin depolymerization	h-cyclodextrin polymer	non-linear amino acid structure	56