Engineering Biomimetic Peptides for Targeted Drug Delivery

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Targeted drug delivery, the ability to target a drug to a specific site of disease, is the leading frontier in the pursuit of better strategies that will allow us to selectively treat diseases with minimal side effects, and peptide functionalized nanovectors are a promising class of targeted delivery vehicles. Biomimetic peptide targeting ligands, peptides that mimic cell binding domains of proteins, can be readily designed to bind a target (for example, an adhesion receptor on the surface of a cell) selectively with high affinity and specificity, and more importantly are molecules accessible by chemical synthesis and relatively compact compared to antibodies and full proteins.

PEPTIDE FUNCTIONALIZED LIPOSOMES

Liposomes are the most extensively studied drug transport systems to date, with a number of non-targeted liposome delivery systems already FDA approved and being used in a clinical setting. Liposomes can range from approximately 50 nm to microns in diameter, although diameters 100-200 nm are often desirable for drug delivery applications. "Stealth" liposomes, also often referred to as sterically stabilized liposomes, have short polyethylene glycol (PEG) polymer strands attached to a fraction of the hydrophilic lipid headgroups. These PEG chains form a polymer brush on the surface of the liposome that, through steric repulsion, resist protein adhesion and therefore clearance by the reticuloendothelial system (RES). Today, one ongoing area of effort in liposome drug delivery research involves conjugating ligands, such as peptides, onto "stealth"

liposomes to confer active as well as passive targeting to these drug carriers. Like liposomes, polymeric drug delivery vectors serve to encapsulate their cargo and shield it from degradation and clearance from the body. Also many of the same peptide targeting ligands are conjugated to polymeric delivery vehicles.

There is an inherent conflict of design for most targeted delivery nanovectors, in that the surface is typically coated with a polymer brush to inhibit protein adhesion and therefore clearance by the RES, while at the same time ligands are installed on the surface to promote targeted adhesion. Peptides have many advantages as targeting ligands: they are small molecules, can be efficiently chemically synthesized, can achieve high specificity, and are easily integrated into liposomes as peptide-amphiphiles (Tu et al., 2004). Peptide ligands can be designed to mimic protein binding sites, or they can be identified from large peptide libraries with selection techniques such as phage display.

Today there are a multitude of different peptide ligands for a wide range of target receptors, each exhibiting varying binding specificity and affinity. Liposomes have been functionalized with different peptides such as a peptide named SP5-52 that binds to tumor vasculature; the basic fibroblast growth factor peptide (bFGFp) that specifically binds to FGFR expressing cells such as melanoma, breast cancer and prostate cancer; the pentapeptide YIGSR, derived from the glycoprotein laminin, that was shown to bind with high affinity to the laminin receptor over-expressed in human tumor cells; the NGR and APRGP peptides that have been used as potential targeting moieties against tumor vasculature, and others (Pangburn et al., 2009).

A different strategy for the delivery of therapeutic loads to target cells is the use of peptide sequences derived from Protein Transduction Domains (PTDs), also called cell

penetrating peptides (CPPs). PTDs are short peptide sequences that mediate translocation across the cell membrane (Torchilin 2008). Examples of these PTDs include the Antennapedia peptide (Antp), the HIV-TAT (Transactivator of Transcription) peptide, poly-arginine peptides, and penetratin (Breunig et al., 2008). Cell uptake by both these peptides appears to bypass the endocytic pathway, and there are different theories on the mechanism of CPP mediated uptake (Torchilin 2008). The TAT peptide derived from HIV-TAT is one of the more frequently used CPPs (Torchilin 2008), and has been conjugated to liposomes to aid intercellular delivery of therapeutic loads (Tseng et al., 2002; Torchilin et al., 2003; Marty et al., 2004; Kale and Torchilin 2007; Oba et al., 2007). For example, Kale and Torchilin formulated a stealth liposomal delivery system with TAT conjugated on the surface of the particles. The liposomes were delivered to the tumor sites by the EPR effect and lost their PEG coating in the low pH tumor environment thus exposing the underlying TAT peptides, which were then able to mediate transport into the tumor cells (Kale and Torchilin 2007).

Another class of targeting peptides are the fusogenic peptides. The capacity of fusogenic peptides of natural (e.g., N-terminus of hemagglutinin subunit HA-2 of influenza virus) or synthetic (e.g., WEAALAEALAEALAEHLAEALAEALEALAA (GALA), or WEAKLAKALAKALAKHLAKALAKALKACEA (KALA)) origin has been exploited for the endo-/lysosomal escape of several drug delivery systems (Plank et al., 1998; Li et al., 2004). These peptides assume a random coil structure at pH 7. Acidification triggers a conformational transition, that allows their subsequent interaction with the lipid membrane, resulting in pore formation or the induction of membrane fusion and/or lysis (Breunig et al., 2008). Incorporation of synthetic membrane-active peptides

into delivery systems has been found to enhance intracellular delivery of drugs including oligonucleotides, peptides, or plasmid DNA (Breunig et al., 2008).

Liposomes Functionalized with Collagen-Mimetic Peptides

Collagen-mimetic peptides have been developed to target the CD44 receptor. CD44 is over-expressed in many tumor cells, and ligand binding causes endocytosis (Tammi et al., 2001; Jiang et al., 2002). Specifically, CD44 in metastatic melanoma is in the chondroitin sulfate proteoglycan (CSPG) modified form (Naor et al., 2002). CD44/CSPG receptors bind to a specific amino acid sequence from type IV collagen $\alpha1(IV)_{1263\text{-}1277}$ (GVKGDKGNPGWPGAP), called IV-H1 (Chelberg et al., 1990; Fields et al., 1993; Lauer-Fields et al., 2003), and more importantly, binding is highly dependant on the triple helical structure of the sequence, as well as CD44 being in the CSPG modified form (Fields et al., 1993; Malkar et al., 2002; Lauer-Fields et al., 2003).

The IV-H1 peptide sequence was functionalized with different dialkyl tails to create collagen-like peptide-amphiphiles. Results showed that while the IV-H1 peptide did not exhibit any positive ellipticity similar to a polyPro II helix, the peptide-amphiphiles investigated were all in triple-helical conformations (Yu et al., 1996). More over, the triple-helical peptide-amphiphiles were very stable. This was an example where the self-assembly of the hydrophobic tails of the peptide-amphiphiles served to align the peptide strands and induce and/or stabilize the three-dimensional structure of the peptide headgroup into triple helices, giving rise to protein-like molecular architectures (Figure 1).

Previous studies have shown that a peptide-amphiphile with a peptide headgroup [$(GP\text{-}Hyp)_4$-GVKGDKGNPGWPGAP-$(GP\text{-}Hyp)_4$-NH_2] mimics the $\alpha1(IV)_{1263\text{-}1277}$

sequence in structure and function, and specifically binds to CD44/CSPG (Yu et al., 1996; Yu et al., 1998; Yu et al., 1999; Lauer-Fields et al., 2003). This peptide-amphiphile was incorporated into a stealth liposome, was targeted to M14#5 metastatic melanoma cells, and promoted specific ligand/receptor interactions where as non-targeted liposomes showed no binding (Rezler et al., 2007).

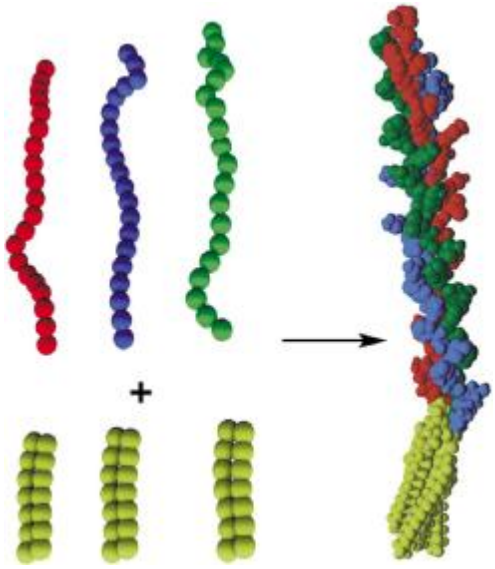

FIGURE 1 Structure of a peptide-amphiphile incorporating triple-helical protein-like molecular architecture. Long-chain dialkyl ester lipid tails are connected to linear peptide chains. The tails associate by hydrophobic interactions, inducing and/or stabilizing the 3-D structure of the peptide headgroup. Triple-helical molecular architecture is stabilized in the peptide-amphiphile. Source: (Tirrell et al., 2002).

PR_b-Functionalized Liposomes

Peptide ligands based on the tripeptide RGD (Arg-Gly-Asp) sequence are some of the most widely used in targeting research. The RGD sequence, located in the 10th type III repeat of the fibronectin molecule, originally identified as a cell binding site in the extracellular matrix protein fibronectin, has been used as a targeting moiety in numerous occasions. Though RGD has been used with some success as a targeting moiety against

integrins, it does not have the same adhesive properties as native fibronectin (Akiyama et al., 1995; Yang et al., 2001; Garcia et al., 2002). A synergy amino acid sequence, Pro-His-Ser-Arg-Asn (PHSRN), located in the 9th type III repeat of fibronectin (Figure 2) was identified and shown to improve binding affinity and be critical for specificity to the $\alpha_5\beta_1$ integrin (Aota et al., 1994; Leahy et al., 1996). Various targeting moieties incorporating both the RGD and PHSRN sequences have been tested, however most of these designs were unable to achieve the cell adhesion densities supported by native fibronectin over similar time scales (Kao 1999; Aucoin et al., 2002; Kim et al., 2002; Benoit and Anseth 2005; Petrie et al., 2006). Mardilovich and Kokkoli postulated that for a small peptide to effectively mimic the $\alpha_5\beta_1$ binding site of fibronectin the primary (RGD) and synergistic (PHSRN) binding sequences must be connected by a linker that approximated both the distance and hydrophobicity/hydrophilicity between these sequences in fibronectin resulting in a neutral linker (Mardilovich and Kokkoli 2004). While previous efforts attempted to match the distance between the RGD and PHSRN sequences, they did not pay particular attention to the hydrophilicity/hydrophobicity of the linker. The efforts of Mardilovich and Kokkoli culminated in the design of a biomimetic peptide, named PR_b, that is now well established as a close mimic of the $\alpha_5\beta_1$ binding site in fibronectin and a highly effective and specific targeting peptide (Mardilovich et al., 2006). PR_b has been shown to bind specifically to the $\alpha_5\beta_1$ integrin, and to promote cell adhesion more effectively compared to similar peptides with hydrophobic or hydrophilic linkers and even fibronectin (Craig et al., 2008). When attached to a 16-carbon dialkyl tail to form a peptide-amphiphile (Figure 2) PR_b can be easily incorporated into a liposome.

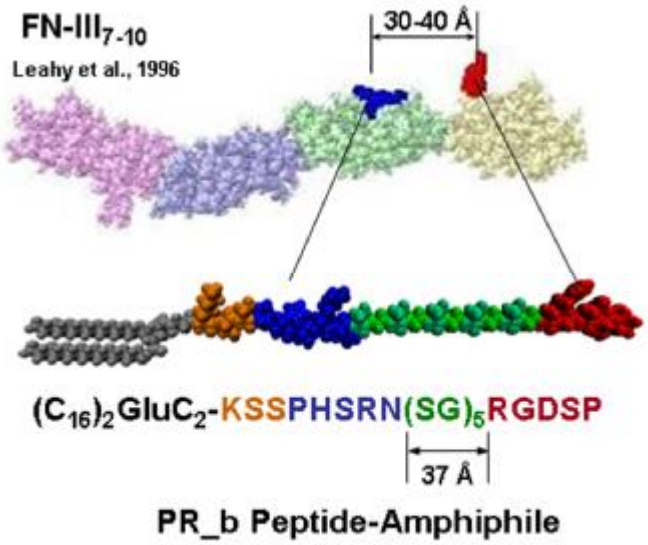

FIGURE 2 The four repeats of the fibronectin (FN) fragment III_{7-10} are shown: far left repeat for III_7 to far right for III_{10}. The synergy site PHSRN is in the III_9 repeat and the GRGDS in the III_{10}. The PR_b peptide has four building blocks: a KSS spacer, the PHSRN synergy site, a $(SG)_5$ linker, and the RGDSP binding site. A schematic of the PR_b peptide-amphiphile is shown. When the PR_b peptide-amphiphile is used for the preparation of functionalized liposomes, the hydrophobic tail is part of the membrane and the peptide headgroup is exposed at the interface.

Recently, stealth liposomes functionalized with PR_b were used for the targeted delivery of therapeutics to colon cancer cells (Garg et al., 2009; Garg and Kokkoli 2010) and prostrate cancer cells (Demirgöz et al., 2008). In these studies, PR_b functionalized stealth liposomes loaded with a chemotherapy agent were more effective than RGD functionalized stealth liposomes or non-targeted stealth liposomes in terms of cell adhesion, internalization, and cancer cell toxicity (Figure 3).

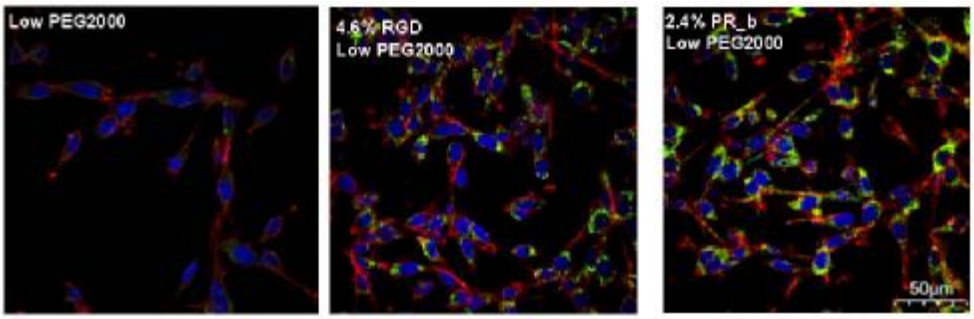

FIGURE 3 Confocal images that show internalization of targeted stealth liposomes to CT26 colon carcinoma cells. The images show the cell membrane, the nucleus, and the drug delivery systems shown between the nuclear region and the cell membrane. Different formulations with low densities of PEG2000 (2-3%) were incubated with CT26 at 37ºC for 24 hr. The scale bar is 50µm for all images. Source: Adapted from (Garg et al., 2009).

CONCLUSION

A wide range of peptide targeting ligands have been studied with some: RGD, TAT, NGR, and bFGF finding much more frequent use in different delivery systems, both liposomal and polymeric. There are numerous advantages of installing a targeting ligand onto the surface of a delivery nanovector with countless researchers confirming increased cellular adhesion internalization and targeting.

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