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A comparison of peptide and folate receptor targeting of cancer cells: from single agent to nanoparticle

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Introduction: There is broad interest in a targeted strategy that delivers a concentrated therapeutic payload to tumor cells, because of the significant potential for improvements in therapeutic outcomes and reduction of side effects if therapeutics can be delivered only to diseased tissue.

Areas covered: This review describes how the coupling chemistry and surface charge effects of peptide labeling in nanoparticle drug delivery strategies have proved difficult to control, resulting in many studies that use folate instead. However, the successful peptide targeting of structural, hormonal, cytokine and endocrine receptors in the delivery of therapeutic and diagnostic radionuclides provides a strong indication that it is worth finding methods to synthesize peptide-targeted nanoparticles.

Expert opinion: Chemical conjugation to peptides reduces colloidal stability, which is a limiting factor in the development of targeting nanoparticles. Mechanistic studies are needed in order to develop peptide targeting for nanoparticles to rival the selectivity that has been achieved with the small molecule folate. Although most of the work so far has been done using gold nanoparticles, biological and polymer nanoparticles are more colloidally stable and present enormous opportunities for coupling to peptides.

Keywords: antibody, caveolar uptake, cell-penetrating peptide, clathrin-mediated endocytosis, colloid, cytokine, dendrimer, endosomolytic peptide, hormone, liposome, macropinocytosis, phage display, poly(ethylene glycol) polymer

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1. Introduction

A major goal for cell targeting is to internalize a therapeutic agent, which can be a drug, radionuclide, or nanoparticle carrier. One may contrast the single-agent approach with a nanoparticle approach in order to understand key issues that confront a targeting strategy. In practice this is seldom done. The nanoparticle field has grown rapidly with little reference to the extensive field of targeted delivery of radionuclides, which is used as a primary example of peptide and metabolic targeting for comparison's sake. The advantages of the nanoparticle approach are: the capacity to carry a high density of therapeutic agents; and multivalent or multifunctional targeting of a cell membrane. On the other hand, the single-agent peptide targeting approach has the following advantages: small size permits diffusion to all tissues; stability is relatively high; and synthesis is relatively easy to control. The juxtaposition of large size and multivalency, on the one hand, as advantages for the nanoparticle field, must be weighed against solubility and lack of a colloidal stability problem on the other, for single-peptide targeting agents. There are several dimensions to the consideration of the role played by peptides in targeting. Perhaps the most important is the response of the immune system.

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All foreign targeting vectors are subject to clearance by the reticuloendothelial system (RES), in which uptake by the liver and spleen plays a major role. In addition, particles > 100 nm are removed from the bloodstream by macrophages. Peptides can also be removed from circulation by the RES or, perhaps more commonly, hydrolyzed by blood proteases, which leads to short circulation times. The origin of short circulation times is different in each each, but the prolongation of circulation time is considered to be an important technological hurdle in all targeted delivery. Nanoscale delivery systems have the potential for long circulation if appropriate surface functional groups are used, the most common being poly(ethylene glycol) (PEG). Although nanoparticles hold much promise if properly designed and implemented for cell targeting, the disadvantages of the nanoparticle approach include colloidal instability, the difficulty of consistent or uniform labeling, and the requirements for endocytosis and endosomal escape in the target cell. Therefore, it is worth comparing the success of the single-peptide approach for radionuclide delivery with the nanoscale approach to peptide targeting. One may hope that this comparison helps to push both fields in a fruitful direction. Obviously, there are targeting strategies built on antibodies and larger protein targeting agents. These are considered outside the scope of this review. This review focuses on a comparison between short peptides and small molecules, with an emphasis on folate because of its widespread application [1].

Although it appears reasonable to try to utilize peptide targeting to create a highly selective nanoparticle targeting agent, it has proved challenging because of the fact that chemical modification of a nanoparticle with peptides can destabilize the colloid by alteration of surface charge and hydrophobicity. For this reason, most nanoparticle targeting studies have relied on agents other than peptides. The most widely used are the metabolites folate (molecular mass 441 Daltons) and the iron-carrier protein transferrin (molecular mass ~ 80,000 Da). The focus here is on a comparison folate with peptides as transferrin is a large protein that may more properly be considered in comparison with antibodies or other protein targeting agents. The distinction is made between small multifunctional targeting agents that can be attached to nanoparticles, on the one hand, and antibody, phage or intact proteins, on the other, which are practically 'nanoparticles' in their own right. These targeting agents significantly alter the size and chemistry of a nanoparticle, such that their consideration should be separate from the attachment of targeting peptides and metabolites to a nanoparticle scaffold. As peptides are derived from certain therapeutic antibodies, key clinical antibodies are included in **Table 1**, which provides a list of tumor targets and their specificity. **Table 1** is far from an exhaustive list, but it suffices to exemplify the targeting capabilities of short peptide sequences. In this review, the new field of nanoparticle targeting is contrasted with radionuclide targeting, which has used a range of metabolic and peptide receptors to produce a wealth of imaging data [2] and some therapeutic success. The focus of the review is on understanding the potential benefit of a large number of cell-targeting peptides and the pitfalls for nanometer-scale synthesis using them.

2. Nanoparticle drug delivery: presentation of targeting peptides and metabolites

Multivalent inorganic nanoparticles [3-5], liposomes [6,7], polymers [8], dendrimers [9], peptide-based nanoparticles [10,11], protein shells [12,13] and plant virus particles [14-16] each present a distinct strategy for a targeting vector that can carry a payload to cancer cells. Therapeutic delivery of drugs concentrated in a nanoparticle ideally combines a loading method for the drug molecules with a targeting strategy to deliver those drugs to a specific cell population. The role of the peptide or metabolite (both called ligands in the following) in facilitating cell targeting depends strongly on the presentation of the molecular ligand to the cell surface [17]. The internalization of ligands depends on the specific receptor type targeted, as well as on multivalency, which is defined as the presentation of several targeting agents on a single nanoparticle. The ease of chemical attachment to colloidal gold through thiolate bonding has led to extensive applications dating back to the 1960s [18]. For this reason, most studies even in recent times that document multivalency have been conducted with gold nanoparticles, accounting for their prominence in this review. One can compare a nanoparticle strategy for delivery to that of viruses, which have evolved to deliver their genome to cells. In both cases multivalency leads to stronger binding with a target cell [19,20]. Viruses not only target cell surface receptors, they also escape from the endosome to avoid sorting to the lysosome and degradation. The specific amino acid sequences that disrupt the membrane are known as endosomolytic peptides, which are usually hydrophobic fusogenic peptides in viruses [21]. The challenge for nanoparticle strategies is that all of the required functions of endocyosis, escape and internal targeting must be accommodated in a particle that is colloidally stable.

3. Colloidal stability: the central problem for targeted nanoparticle drug delivery

Nanoparticles are colloids, and therefore their stability is subject to the laws of colloidal stability. Colloidal, and therefore nanoparticle, stability is explained by a combination of Derjaguin–Landau–Verwey–Overbeek (DLVO) theory [22,23] and steric stabilization described by Flory–Krigbaum theory [24], shown in **Figure 1**. DLVO theory describes the competing factors of polarizability and electrostatics to explain nanoparticle stability. The force of attraction resulting from polarizability tends to lead to flocculation and aggregation. However, this force is opposed by mutual electrostatic repulsion of like-charged colloids, which acts to stabilize the suspension (see **Figure 1B**). According to the DLVO theory, which

Table 1. Common peptides for nanoparticle cell uptake applications.

Peptide type	Sequence	Targeting classification	Ref.
Tat	AGRKKRRQRRR	CPP	[56]
Penetratin	RQIKIWFQNRRMKWKK	CPP	[56]
AdnNLS	GGFSTSLRARKA	CD46 receptor, nuclear localization	[137]
AdnCAR	NPVYPYEDES	Coxackie-adenoviral receptor	[137]

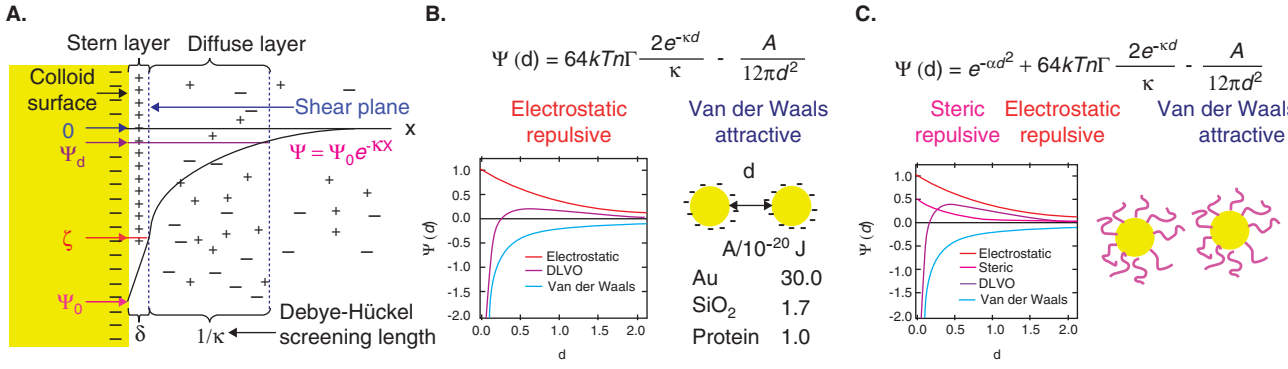

Figure 1. Colloidal stability illustrated in terms of the relevant theories. A. The electrostatic atmosphere of the surface of the nanoparticle is illustrated. The surface charge (here shown as negative) is mitigated by tightly bound ions of opposite charge in the Stern layer. The diffuse layer consists of ions that are attracted to the surface. **B.** The opposing forces of electrostatic repulsion and van der Waals attraction combine to give a barrier that prevents flocculation and aggregation. The Hamaker constant, *A*, in H_2O is given for three relevant materials, Au, SiO_2 and a polypeptide (protein). **C.** The addition of flexible polymers can confer a steric stabilization that can increase the barrier to flocculation.

ignores steric interactions, polarizability is a universal attractive potential in all materials, which is commonly referred to as the van der Waals attraction. The material parameter that governs the magnitude of the attractive term is the Hamaker constant, *A* [25]. The value of *A* ranges from 30×10^{-20} J for Au to $1 - 2 \times 10^{-20}$ J for organic compounds, including proteins and polymers (**Figure 1B**). The Hamaker constant explains why gold nanoparticles (AuNPs) are the least stable of all colloids (except Ag, which has a higher value of the Hamaker constant). The stability conferred by the balance of the attractive and repulsive potentials will be reduced if the surface charge is neutralized. The citrate-coated AuNP in **Figure 2** demonstrates that if the solution pH reaches the isoelectric point (pI), that is, if pH = pI, the surface charge is zero and the particles will flocculate, aggregate, and ultimately precipitate. In the titration of citrated-stabilized AuNPs in **Figure 2**, the zeta potential cannot be measured below pH 6 because of aggregation, which removes the nanoparticles from the solution. Nanoparticles can also be destabilized by increased ionic strength, which shortens the Debye length, $1/\kappa \approx (1\ \text{nm}/I)$ in water, where *I* is the ionic strength. Addition of ions to a solution results in screening of the surface charge in the diffuse layer (**Figure 1A**), which alters the surface potential such that nanoparticles can approach more closely. The net effect is to increase the probability of crossing the barrier shown in **Figure 1B**, resulting in flocculation. This is seen in studies of the critical coagulation concentration (CCC), which is the salt concentration at which the suspension is destabilized [26].

The steric effect involves long-range interactions of polymers attached to the surface of nanoparticles, which has been described quantitatively using Flory–Krigbaum theory. The entropic effect of polymer chains stabilizes a suspension because nanoparticle collisions require greater order in those chains, which is an unfavorable change. The enthalpic effect of polymer chains requires that the solvent (i.e., water in this case) be removed from the polymer chain. If solvation of the polymer is favorable, the solvent is called a 'good solvent' in Flory theory. Such a solvent leads to net stabilization of the nanoparticle by polymer chains. For example, water is a good solvent for most proteins and for the polymer PEG. Steric stabilization shown in **Figure 2** for Au nanoparticles surrounded by bovine serum albumin (BSA) is consistent with the long history of use of BSA as a stabilizer of colloidal gold [27]. As the function of serum albumin is to carry hydrophobic molecules in blood, BSA may have evolved to be a colloidally stable protein. **Figure 2** shows that proteins such as cytochrome *c* or myoglobin do not have this property and are not good steric stabilizers [28]. The effect of PEG on the colloidal stability of Au nanoparticles is comparable to BSA [26,29]. The surface

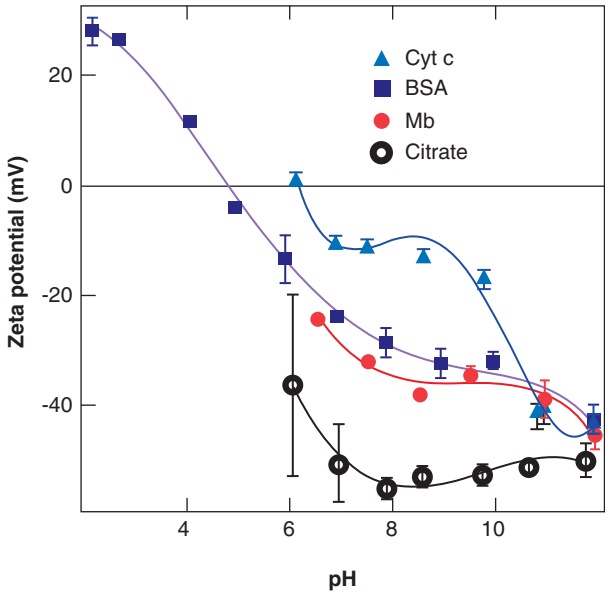

Figure 2. Zeta potential measurement of 20 nm AuNPs coated with various stabilizers. Citrate (black) is the standard coating obtained from the reductive nanoparticle preparation. Cyt c has an isoelectric point near 10. The AuNPs coated with citrate, Mb and Cyt c are unstable below pH 6. The isoelectric point of the citrate-stabilized particle is ~ 4.8. Only BSA stabilizes the particle at and below its isoelectric point.

AuNPs: Gold nanoparticles; BSA: Bovine serum albumin; Cyt c: Cytochrome c; Mb: Myoglobin.

attachment and functionalization of PEG is much easier to control, and recent work has demonstrated stabilization at high salt concentrations (> 1 M) [30,31]. The capability of suspension in salt concentrations > 0.1 M is sufficient for compatibility with cell growth media. PEG can be functionalized by amine termination permitting cross-conjugation of cysteines and amines, as shown later in **Figure 4**, which provides a relevant recent example of successful functionalization and targeting using PEG-coated AuNPs [31,32]. In the liposome field, PEG has played a decisive role both by increasing colloidal stability, and also providing prolonged circulation times. Doxil is a PEG-coated liposome that is used clinically to deliver doxorubicin to cancer patients. However, Doxil is not a targeting liposome, and the current push in the liposome field is to combine steric stabilization with targeting to increase the specificity of drug delivery [33].

There are few systematic CCC measurements to verify the stability of the particles in biological fluids [26]. In 12- or 96-well cell culture plates, in which adherent cells grow to near confluency in the bottom of the plate, there is a danger that the lack of colloidal stability can lead to precipitation and uptake by nonspecific mechanisms, rather than by the desired targeting pathway. Most nanoparticle experiments are conducted at very low ionic strength, and the value of the CCC is often not reported. For this reason the compatibility of nanoparticle formulations with cell growth media is difficult to assess. For many formulations, the ionic strength of Dulbecco's medium (~ 0.1 M), which corresponds to a Debye length of ~ 30 nm, can destabilize a nanoparticle formulation. Blood has a still higher ionic strength. Nanoparticle flocculation and aggregation may play a role in the mechanism of internalization into cells that is often overlooked in the analysis. The effects of aggregation should be considered in experimental design and analysis, in order to control for the possibility of nonspecific uptake [34].

The factors of nanoparticle stability and the consequences for internalization are intertwined with the bioconjugate chemistry used to prepare the formulation. Surface modification of a nanoparticle determines the surface charge and therefore the zeta potential. **Figure 2** gives a simple example in which the stability of AuNPs depends on the pI and steric flexibility of the protein bound to the surface. Although peptides are smaller than proteins, they have a significant effect on both charge and steric stabilization of nanoparticles. Peptide modification of proteins is still the most common method for attachment to solid nanoparticles [35]. The origin of destabilization of AuNPs owing to direct attachment of peptides is still a subject for study, but this obstacle is central to applications in which nanoparticles can achieve their full potential in targeted drug delivery and diagnostics.

4. Bioconjugate chemical attachment of peptides to targeting nanoparticles

Bioconjugate chemistry provides synthetic routes to the chemical attachment of targeting agents. Most studies rely on a limited number of coupling strategies, the most important of which are shown in **Figure 3**. Chemical modification of folate and amino acids starts with consideration of the available functional groups shown in **Figure 3**. Carboxylate (aspartate and glutamate), amine (lysine) and thiolate (cysteine) are the most widely used functional groups for coupling [36]. Peptide targeting has been validated by radionuclide applications, which is an important point when considering the current difficulties in obtaining similar results with targeted nanoparticles. Targeting peptides for radionuclide application also use the same coupling reactions as applied in targeted nanoparticle synthesis. However, in radionuclide applications, a chelating group or other agent that captures a radionuclide is conjugated to the peptide. Thus, **Figure 4** can serve as a general description that applies to the two fields, nanoparticles and radionuclides, being compared in this review.

Figure 4 shows some of the major routes available for the chemical conjugation of two species, designated X and Y. In the reactions shown in **Figure 4** X represents the peptide that contains cysteines or lysines, the amine or thiol functional groups, shown in **Figure 3B, C**, respectively, and Y represents the chelating agent, drug or polymer/nanoparticle to which that targeting agent is coupled. **Figure 4A, a** shows the Michael

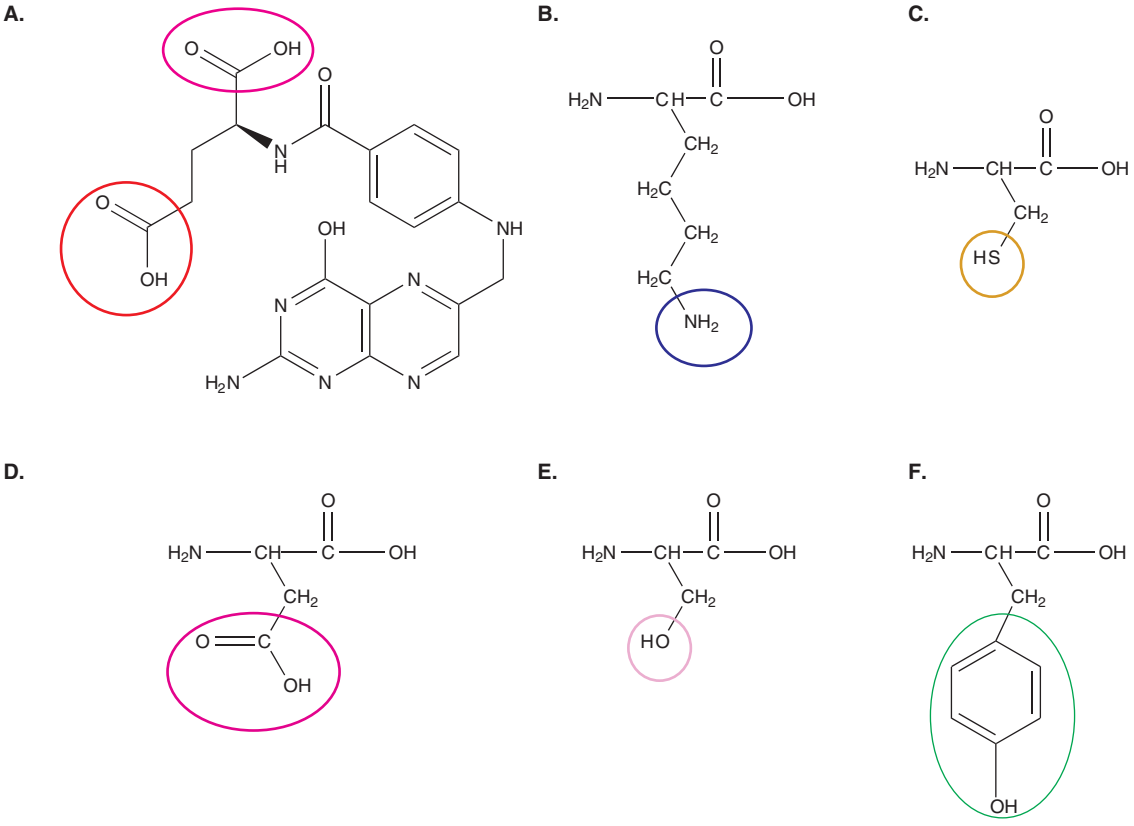

Figure 3. Key functional groups found in proteins and folic acid. A. Folic acid shown with two potential carboxylate groups. The terminal carboxy group is the more reactive. **B.** Lysine has a reactive primary amine below pH 9. **C.** Cysteine has a reactive thiolate. **D.** Glutamate has a reactive carboxy group. **E.** Serine has a reactive hydroxyl group. **F.** Tyrosine has a reactive phenol group.

addition, which is the most common well-controlled coupling of a thiol to succimide to yield a covalent bond. By contrast, coupling of a thiol to thiol, shown in **Figure 4A, b**, to make a disulfide bond is difficult to control with specificity, as all cross-couplings are possible. Moreover, an oxidizing agent is required for this coupling. The coupling of a thiol to a metal (usually Au), shown in **Figure 4A, c**, is widely used in nanoparticle coupling chemistry. The metal itself can reduce the hydrogen atom from R-SH, which facilitates the coupling. Although these chemical coupling reactions are widely used in the preparation of targeting nanoparticles, they are difficult to control. The consequences for nanoparticle stability are difficult to predict because of the possibility of side reactions, the difficulty of complete saturation of binding sites (incomplete reaction), and the effect of the coupling reaction on nanoparticle stability. The reaction shown in **Figure 4D**, known as 'click chemistry' [37], presents an alternative. Although the chemical interactions are distinct from the typical thiol and amine nucleophilic reactions, there are disadvantages as well. First, the reaction must be catalyzed by Cu(I), which can present some complications in subsequent purification of nanoparticles. Second, the reaction yield is not always high. Nonetheless, 'click chemistry' does find application in complex chemical systems [38]. Regardless of the method used for attachment of the peptides, the net result can destabilize the colloidal suspension if the surface charge is reduced or the hydrophobicity is increased during the bioconjugate attachment.

5. Chemical attachment of folate and peptides to nanoparticles: the pH balancing act

Folate has the advantage that it is a small molecule that is relatively soluble, and the disadvantage that the functional groups are limited to the carboxylic acids and a relatively unreactive aromatic amine. The carboxylic acid is sufficiently reactive that folate has been attached to targeting nanoparticles, which include liposomes [6,7], polymers [8], dendrimers [9], quantum dots (QDs) [5], silica NPs [39-41], iron oxide NPs [3], AuNPs [4] and plant viruses [14,15]. Folate coupling is sufficiently facile that it has been applied to coated polyethylene imine (PEI)-coated silica nanoparticles [42]. These recent references are examples of a relatively long

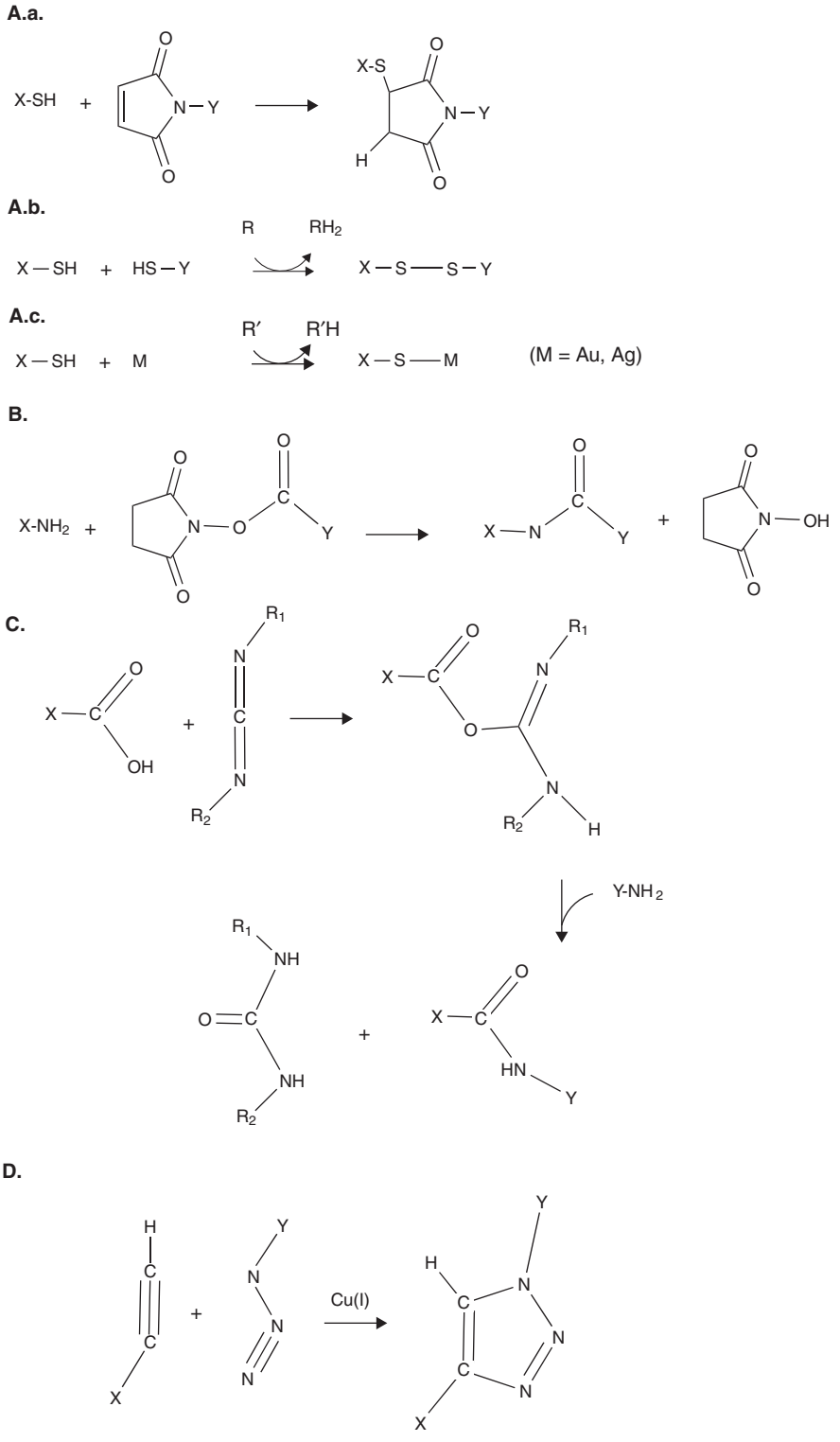

Figure 4. Key coupling strategies in bioconjugate chemistry.

history of folate targeting. Folate targeting has been incorporated into several targeted drugs, some of which are in clinical trials (Endocyte, Inc., West Lafayette, IN).

When folic acid is the targeting agent, it must be chemically modified to present a reactive group. The carboxylic acids of folic acid (**Figure 3A**), glutamate and aspartate (**Figure 3D**) are not reactive towards cross-coupling unless modified to incorporate a leaving group. There are several leaving groups that can be introduced using carbodiimide chemistry. One of the most common leaving groups is the *N*-hydroxysuccinimide (NHS)-ester, as shown in **Figure 4B**. The preparation of the NHS-ester uses carbodiimide chemistry, shown in **Figure 4C**. The activation of carboxylic acids is a key chemical step, widely used in the synthesis of cross-coupling reagents (heterobifunctional linkers), activation of folate, or drugs that contain the carboxylic acid functional group, and even direct modification of peptides or proteins. One common reagent used for activation of carboxylic acids towards chemical coupling is 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). Given the common occurrence of glutamate and aspartate, the direct application to peptides and proteins is relatively rare. Specifically, the widely used coupling of folic acid uses the NHS-ester chemistry. Although these methods are widely used to create targeting peptides and nanoparticles, it is important to recognize that there are limitations in terms of both the coupling efficiency and the stability of the final product that may limit the utility of the method [36]. The optimal pH for these syntheses is a compromise between of the rate of nucleophilic attack by the nucleophile (usually lysine) compared with hydrolysis by hydroxide, which also increases proportionally to the pH. These factors are important because nanoparticle stability requires maintenance of the pH away from the isoelectric point of the particle in order to maximize colloidal stability. Thus, preparation of the nanoparticle targeting agent is a balancing act that requires consideration of nanoparticle stability at each phase of the synthesis of a complete multifunctional targeting agent.

Electrostatic repulsion and steric stabilization are both strongly affected by the attachment of targeting groups that alter the surface charge of the nanoparticle. Although the effects of summed charges can be complicated, it is possible to estimate the net effect of surface chemistry on the charge of a nanoparticle. As folate is negatively charged, the net effect of folate bioconjugation by NHS chemistry (**Figure 4B**) is to reduce the surface by two units of charge. In addition to the negative charge of folate itself, the amine group of lysine is removed by the conjugation reaction, and this removes one charge because the $-NH_2$ group is protonated at physiological pH (pH ~ 7.4). The effect of surface-attached peptides is much more difficult to estimate because of the complication that one must estimate the pI of the peptide and determine how it will interact with other surface charged groups. Practically speaking, this limitation has impeded the application of peptide coupling to various nanoparticles.

6. Discovery and synthesis of cell-targeting peptides and peptidomimetic ligands

In comparing multivalent nanoparticle targeting with single-agent peptides, the various functions of the targeting peptides must be understood to determine the fate of a targeting peptide formulation. Peptide targeting is based on naturally occurring cell signaling, motility or metabolic processes that involve the binding of ligands to cell surface receptors. The result of binding may be to trigger a cell signaling cascade, or to induce cellular uptake by either clathrin-mediated or caveolar endocytosis. Peptides that can target cells can be derived from hormones, cytokines, viral targeting peptides, phage display methods and antibodies [43-51]. Microarray determination of cancer cell genotypes has helped to define potential targets, which are overexpressed cell surface receptors [52]. In addition to these targeting strategies, a class of highly cationic peptides, known as cell-penetrating peptides (CPPs), has been discovered to have the ability to penetrate cells [53-56]. This discovery led to experimentation with systematic modification of the cationic peptide sequence to test the mechanism. CPPs do not show a high degree of cell-type specificity and have been considered as general targeting agents for all cells. They are listed along with the well-studied adenoviral targeting peptides in **Table 1**, which also lead to endocytosis in a range of tissues. One may contrast these peptides with the tumor targets given in **Table 2**. Given the vast number of peptides, this list is representative rather than exhaustive. The classes of receptors include growth factor receptors (EGFR [57], HER-2 [58-61], fibroblast growth factor receptor (FGFR) [62-64]), structural receptors (integrins [65,66], cadherins [67]), hormonal receptors [68-72] and cytokine receptors (e.g., CXCR4) [73,74]. The tumor-targeting peptides are specific for certain cell types in the case of hormonal and cytokine peptides, or are overexpressed in a cancers more generally, in the case of the structural targets integrin [65,66] and cadherin [67]. The growth factor receptors have provided the most successful examples of targeting, with specific antibodies now in the clinic. In the context of this review, the authors are interested in the peptides that are derived from those antibodies or mimic their action; these peptides are listed with the antibodies in **Table 2**.

Each type of peptide provides interesting possibilities for the development of targeted agents capable of entering cells with specificity for tissue and disease. The key feature in these strategies is the ability to modify chemically the peptide for attachment to a therapeutic radionuclide, drug, or nanoparticle that can carry multiple agents. Common chelating agents for radionuclides include tetra-azacyclododecane tetra-acetic acid (DOTA) and diethylene triamine pentaacetic acid (DTPA), which are readily attached to a peptide by one of the methods shown in **Figure 4** [69]. Specificity of coupling to a peptide is usually ensured by using a sequence that has a single cysteine or lysine. However, unlike the targeting peptide case, nanoparticle conjugation is typically more

Table 2. Significant tumor receptors and corresponding targeting agents.

Receptor	Sequence or name	Targeting agents	Target	Ref.
Folate	Folate	Molecule	Any	[1]
EGFR	Panitumumab	Antibody	Colon, prostate	[57]
	GE7, NFWGYIGERPQYRDL	Peptide		
HER-2	Traustuzumab	Antibody	Breast	[58-61]
	FCGDGFYACYMDVK	Peptide		
	MYWGDSHWLQYWYE	Peptide		
FGFR	KRTGQYKLC	Peptide	Breast, bladder, prostate	[62-64]
CXCR4	RR-*o*-CYCiRDKPYRCiCR	Peptide	Breast	[73,74]
	GYRR-Nal	Peptide		
Integrin	Vitronectin	Peptide[§]	Endothelial	[65,66]
	RGD-repeat	Peptide		
Cadherin	HAV-containing	Peptide	Melanoma, epithelial, others	[67]
Membrane-bound guanylate cyclase	Enterotoxin, SSNYCCELCCNPACTGCF	Peptide[§]	Colon	[72]
		Peptide*		
Gastrin-releasing peptide receptor	Bombesin, EGNQWAVGHLM	Peptide[§]	Prostate	[71]
		Peptide*		
Somatostatin receptor	Octreotide, (D)FCY(D)WKTCT	Peptide*	Pancreas, endocrine, breast	[68]
	Pansomatostatin, RFF(D)WKTF	Peptide*		
		Peptide[§]		
		Peptide*		
Melanocortin type 1 receptor	α-Melanocortin, Ac-Nle-DH(D)FRWG[‡]	Peptide[§]	Melanoma	[84]
		Peptide*		
Neurotensin receptor	Neurotensin, ELYGNKPRRPYIL	Peptide[§]	Pancreas, breast	[69,70]
		Peptide*		

*Radioisotopically labeled targeting.
[‡]Chelating group on the C terminus.
[§]Naturally occurring peptide.

difficult to control. The coupling to a nanoparticle surface can involve direct coupling to the metal as in the case of thiols shown in **Figure 4A**, **c**, and coupling to surface-attached amines or carboxylic acids using NHS and EDC chemistry, shown in **Figure 4B**, **C**, respectively. In addition to direct attachment of the peptide, proteins can be attached to a nanoparticle and used as a scaffold for peptide attachment.

One may contrast the peptides typically used for radionuclide targeting with those that have been used in multivalent applications. Radionuclide targeting has relied on hormones including somatostatin and related peptides. Multivalent targeting has relied on viral targeting sequences [19], for example, adenovirus sequences in **Table 1**, structural peptides such as integrin and cadherin binding [75-78], and growth hormones, which rely on dimerization for action. CPPs have found application both as a single peptide agent to promote internalization of covalently attached drugs, proteins and oligonucleotides [79], as well as for nanoparticle targeting [17,80,81].

7. Peptide and metabolic targeting of radionuclides

Delivery of a single agent, such as a radionuclide, is dependent on attachment of a chelating group (e.g., DOTA or DTPA) or chemical modification of the peptide to incorporate the radioactive atom [2]. The great challenge in the radionuclide field is efficient labeling because often the yield of radiolabel binding to a chelator (or other site) is low. The workhorse of the radioactive diagnostic field, technetium-99 m, has poor affinity for most chelators, leading to a low labeling yield. For this reason nanoparticle labeling by radionuclides is a growing field of application [2]. Metabolic targeting of radionuclides is a relatively established field, with the most prominent example being F-18-2-fluoro-2-deoxyglucose [82]. Other metabolite radiolabels include C-11, F-18, I-131, which can replace either naturally abundant carbon or hydrogen atoms in the structure [2]. It is perhaps ironic that despite the wide use of folate as a targeting agent in the nanoparticle field, it is still relatively underutilized in the radionuclide field [83]. Metabolic receptors are present in abundance relative to hormone, cytokine and structural receptors, which has led to the application of radionuclide agents in the diagnosis and treatment of many cancers. Greater specificity for particular cell types and cancer phenotypes can be achieved using peptide targeting, with labeling of In-111, Ga-68 and Cu-64 by binding to DOTA, DTPA and other multidentate chelators that can be chemically conjugated to peptides [2].

The earliest examples of a targeting peptide that can deliver a radionuclide are the somatostatin-targeting peptides [68], which are listed in **Table 2** among other hormonal peptides that target specific tumor types [68,70-72,84]. The wide range of neural, digestive, endocrine and other peptides provides

specificity for tumors in these tissues. Peptide therapy can use a multifunctional approach in a single therapeutic agent, such as the strategy known as the hunter-killer approach, which delivers pro-apoptotic peptides to cells [85]. The significance of these results is that they give an impetus to using peptide targeting in nanoparticles. The presence of multiple binding sites on nanoparticle surfaces may lessen disadvantages of low labeling yield and protease digestion of peptides. However, successful coupling of peptides with stable nanoparticle formulations will require discovering chemical methods of attachment that are compatible with colloidal stability. Beyond the issue of stability, one can consider the trade-off of size versus multivalency as the central issue in comparisons of peptides with nanoparticle formulations. The principal advantage of peptides is their rapid diffusion into the tumor [69]. Although this advantage is removed if they are conjugated to a nanoparticle, the advantage of nanoparticle targeting is multivalency and avidity of binding to the tumor site. Avidity is the effect of multiple binding sites, which can increase the effective equilibrium constant to the power of the number of binding sites. If a single binding site has an equilibrium constant, K, then a multivalent macromolecule with N equivalent binding interactions has a significantly enhanced equilibrium constant of K^N, in the simplest approximation. This simple view has been developed further in recent theoretical studies of multivalency [20]. In considering the trade-off between the single-agent and multivalent targeting vectors, it is still not clear whether penetration or avidity will lead to the greatest efficacy of treatment.

8. The role of targeting peptides in nanoparticle approaches to cancer treatment

The requirement for a unique point of attachment is a factor that renders the peptide conjugation chemistry inherently more complex than attachment of a small molecule such as folate. Despite the difficulties, there are some recent examples of peptides that have been used in nanoparticle studies. A cationic PEI polymer for DNA delivery was attached to a hydrophobic peptide WIFPWIQL [8] that homes to the cancer antigen glucose-regulated protein (GRP-78) or to the RGD sequence, which is an integrin-binding peptide [86]. Although antibody-targeted liposomes have played the largest role in targeted delivery, there are several recent studies that show the potential efficacy of targeting peptides attached to liposomes for targets such as fibroblast growth factor receptor [63] and aminopeptidases A and N [87,88]. The extensive literature on murine and other animal studies using liposomes provides a relevant guide for studies of tissue distribution. For example, it is noteworthy that a positively charged peptide similar to those widely used as CPPs [53-56] actually localizes to the heart to a significant extent [89]. Until quite recently, attachment of peptides to QDs had been limited to streptavidin-labeled QDs, which can bind biotinylated peptides. The intrinsic incompability of the hydrophobic core of the QD has required manipulation in order to permit the attachment of an NHS-ester for peptide attachment [90,91]. Bombesin has been attached to iron oxide particles using click chemistry for targeting of cancer cells [38]. Targeting peptides have been attached or genetically engineered into plant virus protein shells [12,16] and other protein cages [13]. Positively charged peptide nanoparticles have been used to deliver siRNA oligonucleotides and drugs using the CPP strategy for targeting [10,11].

Owing to the ease of the surface chemistry, AuNPs have been by far the most widely studied for peptide-targeting applications on the nanometer scale. When synthesized using the most common preparations [92,93], the AuNPs are coated with citrate ion, which confers a significant negative charge. Although citrate is easily displaced by the strong Au–S bond (**Figure 4A, c**), the addition of typical peptides to AuNP surfaces is sufficiently destabilizing owing to surface electrostatic interactions that direct attachment of peptides was achieved only after research was carried out to find a stable peptide sequence. Direct coupling of peptides yields stable nanoparticles only for particular sequences, such as the CALNN sequence discovered by Levy and co-workers [94]. The work with these directly coupled peptides (**Figure 4A, c**) underscores the difficulty of balancing nanoparticle stability with targeting function. Alternative sequences are destabilizing or can even promote aggregation such as amyloid plaques [95]. Peptides have also been designed that specifically bind to amyloid plaques (e.g., CDLPFF) [96]; however, they are hydrophobic and unstable in solution. There are many examples of alternatives that have been sought using mixed peptide cocktails [97], glycine-cysteamine capping [98], including cationic peptides such as polyarginine and polylysine [99-101]. The therapeutic peptide Kahalalide F has a positive charge and may act as a CPP [102], however the stability of this formulation was not determined. The only available example of nanoparticle targeting by a typical hormonal peptide from the radionuclide-labeling field (**Table 2**) is the application of octreotide to 20 nm AuNPs [103], which binds to the AuNP surface by an unknown mechanism. It is clear that there is also some aggregation in this preparation [103]. Where data are available, peptide formulations are only marginally stable at physiological pH unless PEG is used as a stabilizer or the sequence CALNN is used [94]. PEG consists of ethylene glycol units (-CH2CH2O-), which are $\sim \ell = 3.5$ Å in length. If one assumes that these are units in a Gaussian chain then the diameter of a PEG_N is $d = \sqrt{N\ell}$, where N is the number of monomer units. For example, PEG_{25} forms a Gaussian chain 17 Å in diameter.

An alternative approach for the production of stable functionalized AuNPs is synthesis using a cationic stabilizer known as a tiopronin [104]. Tiopronin-stabilized particles tend to be quite small (< 3 nm), which increases their colloidal stability but limits their surface. The functionalization of 2.8 nm tiopronin-stabilized AuNPs by cationic peptides such as CCPs was demonstrated using the EDC reagent

(**Figure 4C**) followed by the formation of an NHS-ester (**Figure 4B**) [105]. Recent work using starch as a stabilizer during synthesis [106] or PEG molecules as steric stabilizers [30,32,81] provides a potential path forward for application of AuNPs.

Owing to the limited colloidal stability, there has been limited application of AuNPs *in vivo*, primarily as hyperthermic agents in cancer treatment. Polymer nanoparticles have higher colloidal stability owing to a lower Hamaker constant, but there are limited applications at present. Liposomes have found clinical application, but only for nonspecific tumor targeting by the enhanced permeability and retention effect. In all of these cases, targeted nanoparticle applications are at an early stage. The recent literature has emphasized the importance of PEG and other linkers in presenting multivalent targeting peptides, stabilizing the colloidal formulation and prolonging the circulation time.

9. Routes to cell entry: measuring success in cell targeting

Cell targeting inherently involves interaction with the cell membrane, with the goal of cellular uptake to deliver a therapeutic payload to the cell. Nanoparticle investigations have drawn inspiration from viruses, which have evolved mechanisms to target receptors, escape from endosomes and translocate to the nucleus. Similarly, an effective nanoparticle cell-targeting strategy provides a route for translocation into the cytoplasm and then to an intracellular location. Three major routes to internalization in cells that may be exploited by targeting peptides can be distinguished. These are clathrin-coated pit endocytosis, caveolar endocytosis and macropinocytosis [34,107-109]. Phagocytosis is also a known process that involves the uptake of viruses or even micrometer-sized objects in cells when they have been coated with IgG antibodies or complement as part of the immune response [110-112].

The uptake of negatively charged particles such as DNA-coated particles has a low yield and probably occurs by a phagocytic mechanism [34]. Phagocytosis normally occurs in cells in the immune system as part of the RES. Indeed, in a nanotechnology strategy that uses targeting peptides, it is important to understand phagocytosis because it is a process that governs clearance of targeting agents in the > 100 nm size range from the blood, leading to a short circulation time. In addition to increasing colloidal stability, PEG has the added benefit of increasing the circulation time of nanoparticles in blood circulation [113]. Outside the cells in the RES, phagocytosis is relatively unimportant under normal circumstances. However, cells may be stressed by the presence of a high concentration of nanoparticles, particularly if these particles have settled on the cells owing to aggregation. The result may be induction of a phagocytic response, which leads to nonspecific uptake. Interpretation of the results of *in vitro* cell targeting depends on the methods used to control for each of three main methods of internalization in healthy cells, receptor-mediated endocytosis, caveolar uptake and macropinocytosis. The effects of coatings on gold nanoparticles on uptake and cytotoxicity have been reviewed recently [114].

9.1 Receptor-mediated endocytosis

Metabolic, motility and growth factor receptors are examples of cell surface receptors that are endocytosed by the formation of clathrin-coated pits, which leads to formation of endosomes [115]. This pathway can be referred to as clathrin-mediated endocytosis (CME) [116], but has also long been known as receptor-mediated endocytosis (RME). The CME route of cell entry results from interactions of these sequences with the adaptor proteins (AP-2 and AP-180) that act as an intermediate step in the recruitment of clathrin to make a coated pit. Clathrin ultimately creates a spherical vesicle that eventually breaks away from the plasma membrane. The fate of cell surface receptors in the CME pathway is determined by signaling peptide sequences on the cytoplasmic domain of the receptors [115]. The cytosolic signaling sequences for the recruitment of adaptor AP-2 have been characterized for a large number of metabolic receptors (transferrin receptor [TR], folate receptor [FR], etc.), growth hormones (EGFR, FGFR, etc.) and structural receptors responsible for cell motility. Metabolic receptors FR and TR are sorted to recycling endosomes, which return to the plasma membrane. Growth hormone and structural receptors are usually targeted for destruction and are sorted into lysosomes, where the contents of the vesicle are degraded by enzymes. A salient feature of the endosome environment is that the pH of endosomes is lowered to pH < 6.0 by proton pumps and lowered further still in lysosomes. Typically, investigators in the nanotechnology field have tested for CME by lowering the temperature to 4°C or the by application of chloroquine, to disrupt the endosome. Lowering the temperature is not highly specific because it will shut down all activated processes. As chloroquine disrupts the endosomal membrane and leads to release of the contents into the cytosol, it is specific for CME. Ideally, agents such as chloroquine would be studied by the use of live cell imaging to determine whether uptake occurs by means of a clathrin-coated pit mechanism.

Although there are natural ligands and peptides that target these receptors, viruses have evolved to target specific receptors, leading to uptake into endosomes followed by endosomal escape by membrane fusion peptides triggered by the low pH. Examples include influenza hemagglutinin [53] and parvovirus A [117]. However, the coiled-coil motif responsible for pH-triggered membrane fusion is so common that it has formed the basis of the software Multicoil [118]. Peptides that promote endosomal escape have been an active area of research that has led to the prospect of cell targeting that will reach the cytoplasm [21]. Membrane fusion peptides have been used on nanoparticles [80], but pH triggering is difficult to attain with small peptides. A major issue with this approach is that the hydrophobic nature of the exposed membrane fusion peptide lowers colloidal stability, unless

stabilized by PEG as a majority species [81]. The targeting of cells by the adenoviral fiber peptides and endosomolytic peptides has been widely used for CME in conjunction with cationic peptides to coalesce genomic DNA into a nanoparticle [54,119]. However, stability issues have hampered the application of such peptides in AuNPs [26,29,77], which has led to the use of the CALNN sequence, which is one of the few known to stabilize AuNPs [81,94,120]. However, the CALNN sequence is not a targeting or endosomolytic sequence, and its stabilizing property can be overwhelmed by added functional peptides on the surface of a nanoparticle.

Transiting the endosome can result in loss of function of a cargo owing to the harsh endosomal environment [35]. Nanoparticles may be degraded within the endosomal compartments through peptide cleavage by the protease cathepsin L [121]. An alternative approach is to avoid the endosome by using other targets on the cell surface that lead to uptake by other routes, such as caveolae internalization or macropinocytosis.

9.2 Caveolar uptake

Studies of interleukin-2 (IL-2)-mediated internalization led to the recognition that not all viral endocytosis proceeds by means of the clathrin-coated pit mechanism [122]. Caveolae are invaginations of the plasma membrane that are rich in the protein caveolin-1 associated with lipid rafts in the cell membrane. Lipid rafts are cholesterol-rich domains that contain caveolin-1 and several signaling transmembrane proteins. Viruses that promote the formation of lipid rafts can be internalized in caveolae. The SV40 virus can internalize via caveolae [123], although this is not an exclusive pathway [124]. There is some overlap in the receptors that promote uptake by the clathrin and caveolar mechanisms. Endocytosis of cell surface receptors such as transforming growth factor beta (TGF-β) appear to occur by both clathrin-coated pit and caveolar pathways, which suggests a role for regulation of the function of morphogenetic receptors based on the internalization pathway [109,125]. These alternative pathways depend on the cell type and may have different expression in cancer cells. Caveolar endocytosis is inhibited by filipin III, cyclodextrin and a synthetic lipid with non-natural stereochemistry, β-D-lactosyl-*N*-octanoyl-L-*threo*-sphingosine. The reduction of caveolae in oncogenically transformed cells is a known phenomenon that may have consequences for drug delivery strategies [126]. Live cell imaging suggests that some CPPs are internalized by caveolar uptake, rather than by a general nonspecific mechanism [56].

9.3 Macropinocytosis and phagocytosis

Macropinocytosis and phagocytosis are internalization mechanisms that permit uptake of larger particles up to the length scale of micrometers. The process of macropinocytosis involves the actin cytoskeleton and is often associated with a phenomenon known as 'ruffling', which is an observable change in membrane morphology that occurs prior to the process of engulfing a foreign object. Macropinocytosis is often stimulated by growth factors, such as epidermal growth factor (EGF) [127,128] and granulocyte macrophage colony-stimulating factor (GM-CSF) [129], in cells that express cognate receptors. Micropinocytosis, in which the internalized object < 100 nM, is constitutive in all cell types. Macropinocytosis, in which the internalized object > 100 nM, is limited to a few cell types. In these instances, macropinocytosis appears to be synonymous with phagocytosis. Macrophages and epithelial cells stimulated by growth factors with participation of phosphoinositide 3-phosphate carry out phagocytosis for the purpose of antigen presentation [130]. Dendritic cells are specialized to take up foreign objects by macropinocytosis/phagocytosis for processing and surface presentation to T cells. It has been shown that other cell types can be converted to a dendritic cell type by culturing in GM-SCF and interleukin-4 (IL-4) [130-132]. EGF [133] and platelet factor [134] can stimulate endothelial cells, which are crucial for angiogenesis, the role of macropinocytosis. In any targeting strategy that involves cancer, it is important to recognize that macropinocytosis is upregulated in cancer cells [135,136]. This is important because macropinocytosis provides a nonspecific route for nanoparticle uptake in cells that can compete with targeted delivery. The challenge for the development of new targeting strategies involves determining whether specific uptake can be achieved as observed in viral targeting or for hormonal peptide uptake.

10. The competition between specific cell targeting and nonspecific nanoparticle aggregation

Recently, some systematic studies have been conducted to test nanoparticle internalization in cells as a function of surface composition. Studies of AuNPs with three peptides, Tat, Penetratin and AdnNLS (**Table 1**), combined with PEG [81] have challenged work based on serum-album-stabilized targeting [77] using AdnNLS/AdnCAR peptides [137], and work based on Tat-peptide-labeled particles [105]. Specifically, it was shown that cellular uptake could occur for the citrate-stabilized particle control, which was assumed to occur because of exchange with serum albumin in the growth media. The fate of CPP-stabilized cells is still a matter for debate, and a live-cell imaging protocol has been recommended to avoid artefacts due to cell fixation [56]. The lack of mechanistic correlation for uptake of silica particles labeled with various proteins calls into question the role played by targeting [138]. Thus, the current state of studies is that it is difficult to prove that nanoparticle targeting has occurred as opposed to nonspecific uptake. This weakness arises because of the colloidal instability that can lead to aggregation and precipitation in 96-well plates. Ultimately, an aggregate that settles onto a cell can enter by macropinocytosis, phagocytosis or other mechanisms that are not a result of targeting.

Beyond targeting, endosomal escape is key for useful delivery of a drug cargo to a cell. Endosomolytic peptides have been used in gene delivery, which is basically a nanoparticle approach because the genome is condensed to a nanometer-scale particle using cationic polymers [54]. Althoug these methods function *in vitro*, they are not stable in the presence of serum proteins. Liposomes for gene delivery have also been conjugated to endosomolytic peptides [139]. However, endosomolytic peptides tend to be hydrophobic, which lowers the stability of the formulation. The most desirable approach would be a dynamic transition in the endosome, which is the method used by viruses. This method of achieving endosomolysis is difficult because the viral proteins, for example, hemagglutinin, are relatively large in size.

Targeted delivery is the key to effective use of nanoparticles. The challenge in this field has been to prove that uptake occurs by a specific interaction. Nanoparticle binding to cell surface receptors mimics viral targeting in that both occur on the 10 – 100 nm length scale. Viruses can either bind to the plasma membrane and disrupt it directly using various membrane fusion strategies, or be internalized by one of the mechanisms discussed above. Viruses are colloids too, but they have evolved to achieve a relatively high colloidal stability owing to steric interactions and the relatively low Hamaker constants of organic compounds, often with significant internal water. Much of the data available do not provide a clear demonstration of peptide-mediated targeting by nanoparticles. One must be cognizant of the possibility of precipitation on cells, and other interactions that can lead to nonspecific uptake by phagocytosis. As discussed above, recent mechanistic studies provide a first step towards determining whether nanoparticles have, in fact, successfully been endocytosed. However, the demonstration of cell-dependent targeting with appropriate controls is rare and difficult to corroborate. By contrast, folate targeting appears to meet the criteria of targeted delivery. This is probably a product of the relative ease of making a colloidal stable adduct, and the relative difficulty of attaching multiple targeting peptides while preserving colloidal stability. This central issue in the nanoparticle field can be addressed by greater characterization using dynamic light scattering (DLS), transmission electron microscopy (TEM) and fluorescence (where applicable) combined with live-cell imaging to monitor the fate of particles and prove that the uptake mechanism involves specific interaction with cell surface receptors.

11. Conclusion

Metabolic targeting using the chemical attachment of folate to nanoparticles is still a dominant technique. The principle of folate targeting is that the targeted receptor, FR, is overexpressed in cancer cells, and therefore the delivery of nanoparticles presenting a folate molecule constitutes targeted delivery. The radionuclide field gives a great deal of information about the uptake of hormones, cytokines, growth factors, and so on. However, the radionuclide field also shows that metabolic targeting is far more effective because there is a larger number of receptors for nutrients than for hormones and cytokines. Targeting of cancer is based on the overexpression of certain receptors on cancer cells, which applies to nutrients as well as specific growth factors, hormones and cytokines. The potential for specific delivery is potentially greater for peptide-based targeting if the issues of clearance and attachment chemistry can be overcome.

The use of peptides in the nanoparticle field has been pursued actively, but with more limited success. The major issue is colloidal stability, although synthetic issues and presentation of the targeting sequence have also been challenges. As the receptors targeted by peptides are often slated for degradation in the lysosome, peptide targeting faces the extra challenge of the requirement for endosomal escape. Targeting cellular receptors and endosomolysis are both functions that infectious viruses have evolved to carry out successfully [140]. Thus, a great deal can also be learnt about the design of nanoparticles from the study of endosomolytic peptides and nuclear targeting sequences derived from viruses. A successful formulation must balance the needs for a hydrophobic endosomolytic peptide and a charged receptor-targeting peptide with the requirement that the nanoparticle's isoelectric point not be in the vicinity of pH = 7.4, which is the pH of blood, growth medium and the cytosol. Systematic study of this point is rare. The recent studies that show the best results include bifunctional PEG molecules of intermediate length, that is, PEG_{25} [30,95]. If the PEG is attached to the surface of a nanoparticle at one end of the molecule and presents a terminal amine on the other, it can be conjugated to a peptide by standard bioconjugate methods. However, the length of the linker can lead to crosslinking reactions such that this method must be used with care. Moreover, the accessibility of targeting peptides on the surface of a PEG-coated nanoparticle is not clear. Although targeting has been observed, detailed models showing the solvent exposure of the targeting peptide have not been produced.

Folate targeting is more readily implemented than peptide bioconjugation, and will no doubt continue to be a workhorse for targeting given its relative ease of implementation and efficacy. The success of folate is due to the fact that FRs are widely distributed in all tissues because it is an essential nutrient. The relative success of FR targeting in tumor targeting suggests that overexpression of receptors on cancer cells is sufficient to increase the therapeutic index by enhancing specificity of delivery. If this is true for a nutrient, there is an even greater potential for targeted delivery if investigators can begin to use systematically the large repertoire of available peptides for tumor targeting. There are advantages to nanoparticle drug delivery systems, but these require that targeting be balanced with colloidal stability and sufficiently long circulation times to reach the target. Mechanistic study of uptake is the essential step needed to increase the efficacy of targeting peptides in nanoparticle delivery.

A breakthrough in targeting strategies will be reached only when colloidal formulations are sufficiently stable and controlled in composition to permit mechanistic studies that definitively demonstrate the specific nature of cellular uptake. The use of molecular reagents to discriminate between the clathrin, caveolar and macropinocytotic pathways for cellular uptake is now well established. The complementary technique of live-cell imaging has made great strides in recent years. The combination leads to the possibility of screening *in vitro* cell populations to discern the uptake mechanism and fate of a specific nanoparticle formulation. This approach is far superior to the TEM and fixed cell images that are often presented as evidence that a particular formulation has been internalized by receptor-mediated endocytosis. For example, live-cell imaging has revealed specificity in the CPP field so that CPPs are now viewed as a kind of targeting peptide rather than being a nonspecific, electrostatic interaction with the cell membrane. The attention given to CPPs in the nanoparticle field should be extended to other targeting peptides such as hormonal peptides from radionuclide studies in order to take advantage of the large number of potential targets. Live-cell imaging may also prevent artefacts from nanoparticle aggregation from being mistaken for cell targeting.

12. Expert opinion

How does one reconcile the lack of a well-defined mechanism of peptide-conjugated nanoparticle uptake with the numerous studies of targeting peptides labeled with radionuclides that show an effect of targeted delivery? Peptides appear to confer targeting specificity in radionuclide studies, but it has proved difficult to realize this specificity in targeting nanoparticles. Colloidal stability is the central issue that confronts nanoparticle formulations that needs greater consideration. AuNPs have the longest tradition of all cell-targeting strategies, dating back to applications of 'colloidal gold' starting in the 1960s. As gold is one of the most polarizable materials commonly used in the nanoparticle field, the colloidal stability is a major issue that needs greater attention. Of course, formulations are becoming increasingly refined and characterization and detection have both improved markedly since the earliest years of applications of colloidal gold for cell-targeting experiments. However, experimentation with new formulations has also led to the study of unstable nanoparticle suspensions. As a consequence, some of the *in vitro* cell-targeting studies are potentially flawed because of the extremely low colloidal stability of AuNPs. CCC, zeta potential and DLS data should be obtained routinely in order to control appropriately for factors that may lead to nonspecific interactions with cells. Control experiments and mechanistic studies are needed to prove that nanoparticle targeting is specific rather than simply the result of precipitation onto the cells. Indeed, studies with more complete characterization are now beginning to emerge. From this work, it has become evident that AuNPs sterically stabilized by PEG linkers have the most desirable properties for cell-targeting experiments.

AuNPs have the longest history in the cell-targeting field, but they are far from the only option for cell targeting. Nanoparticles composed of materials with small Hamaker constants will have a smaller driving force for aggregation. For example, silica NPs, liposomes, polymers, dendrimers, self-assembled peptides and protein shells such as a ferritin or plant viruses have all been used in cell-targeting applications. Recent experiments have revealed the advantages of ease of functionalization often ascribed to AuNPs. These materials are intrinsically more stable colloids owing to their lower Hamaker constants (15 – 30 times lower than Au). Self-assembled peptide nanoparticles [10,11] and protein shells [13] such as ferritin [12] or plant viruses [14-16] are newcomers to the nanoparticle field that have great potential for cell-targeting applications. The use of biological materials is a sensible direction because these materials are the most biodegradable, readily functionalized and easily loaded with cargo. Some protein and plant viruses can be manipulated genetically. Plant viruses are inexpensive to produce because they can be grown in plants, yeast, or even in *Escherichia coli*. Plant viruses permit the design of an artificial nanoparticle that most closely mimics the desirable attributes of infectious viruses. The desirable characteristics in a drug delivery system include low leakage of the drug cargo, structural integrity, triggered release in the cytosol, multivalent targeting and capability to escape the endosome. The lessons from targeting studies from AuNPs can be applied to plant viruses and other biological nanoparticles to help start this new field. Given the advantages of internal loading, regular structure and higher colloidal stability, these biological nanoparticles are likely to play a much greater role in the future of peptide targeting.

Declaration of interest

S Franzen declares no conflict of interest and has received no payment in preparation of this manuscript.

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