ChemBioChem

Review
doi.org/10.1002/cbic.202300831

Chemistry Europe
European Chemical Societies Publishing

www.chembiochem.org

Therapeutic Peptides, Proteins and their Nanostructures for Drug Delivery and Precision Medicine

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Peptide and protein nanostructures with tunable structural features, multifunctionality, biocompatibility and biomolecular recognition capacity enable development of efficient targeted drug delivery tools for precision medicine applications. In this review article, we present various techniques employed for the synthesis and self-assembly of peptides and proteins into nanostructures. We discuss design strategies utilized to enhance their stability, drug-loading capacity, and controlled release properties, in addition to the mechanisms by which peptide nanostructures interact with target cells, including receptor-mediated endocytosis and cell-penetrating capabilities. We also explore the potential of peptide and protein nanostructures for precision medicine, focusing on applications in personalized therapies and disease-specific targeting for diagnostics and therapeutics in diseases such as cancer.

1. Introduction

Precision medicine combines multiple interdisciplinary fields across molecular biology, chemistry, materials science, and other fields to produce more accurate methods for treating diseases. Advances in sequencing, characterization of cell compartments and therapeutic targets, in addition to advancements in the pharmaceutical industry, have resulted in increased number of advanced studies and clinical trials. Many of these clinical trials consist of protein and peptide drugs for a variety of diseases such as metabolic, immunological, and hormonal disorders and cancers.[1] About 10% of the drugs in the pharmaceutical market are peptide or protein drugs including 485 entries for peptide drugs in DrugBank[2] and 239 entries in THPdb of FDA-approved peptide drugs.[1] Peptide and protein drugs have significant potential in diverse applications, ranging from sensing and catalysis to therapeutics due to their biocompatibility, flexibility in design through amino acid sequence variations, and unique molecular topologies.[3] More specifically, they exhibit unique attributes such as hydrogen bonding potential, inherent chirality from amino acids, polymorphism[4] and conformational rigidity stemming from peptide bonds.[3] Furthermore, peptides can be derived naturally or synthetically, and examples include recombinant hormones, antimicrobial peptides, antibodies, and recombinant enzymes,[5] in addition to the possibilities for incorporating non-natural amino acids to diversify the chemistry further.[6]

Despite significant advancements in drug development, there are still many barriers for precision medicine. For instance, genomic and transcriptomic data alone are often not sufficiently informative for precision medicine. In the case of cancer, the scarcity of genomic drivers of cancer and inability to target many of these drivers pose a great barrier to effective targeted therapy.[7] In addition, even if therapeutics that target such drivers exist, obstacles due to the therapeutics' unfavorable pharmacokinetics, biodistribution, on- and off-target toxicities, and drug resistance must be addressed.[8] This is reflected on the fact that 90% of the clinical trials never reach the market,[9,10] prompting the conclusion that advances in research and development may not always result in enhanced efficacy.[11] Many of these drugs share limitations such as low solubility, proteolytic degradation, and short half-lives in circulation due to instability or immunogenicity;[1,12] therefore, the lack of proper delivery vehicles majorly restricts the implementation of new therapeutics. For this purpose, functional precision medicine approaches have been used to complement the wealth of omics data available and test and predict therapeutic outcomes.[7] Specifically, research into delivery vehicles, even for currently existing therapeutics has shown great promise as it offers the possibility of modulating a drug's pharmacological parameters without compromising the desired effect on molecular targets.[8] In this review, we focus on peptide and protein nanostructures, highlighting some of the more recent developments. We present nanostructures in diverse architectures that contain peptides or proteins to stabilize and provide additional functionalization to a cargo. Therefore, the peptide or protein could be the cargo itself, but can also be a component of the delivery vehicle where it may have different functions such as in targeting, encapsulation, and improving stability. We discuss how nanostructures, including nanoparticles, liposomes, micelles, and nanofibers, are engineered to encapsulate therapeutic agents and carry them to their specific targets, minimizing cytotoxicity and off-target effects, and enhancing therapeutic efficacy. The use of nanostructures in targeted drug delivery provides remarkable potential for

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202300831

precision medicine by enabling personalized treatments that are tailored to individual patients, resulting in improved therapeutic outcomes and reduced side effects. Here, we will focus on the formation and functionalization of peptide and protein nanostructures, and then discuss how these are utilized in precision medicine with discussion of different types of cargo and targeting strategies.

2. Peptide and Protein Therapeutics

Peptides are composed of amino acids and are functional domains of proteins. Peptides and proteins are found ubiquitously in nature, and both natural and synthetic peptides and proteins have also found extensive use on the benchtop and in the clinic. In 2023, until September, out of 40 drugs approved by the FDA were protein-based therapeutics, comprising more than a third of all approved drugs (recombinant enzymes, peptides, hormones, and monoclonal antibodies) (Table 1), which demonstrate the current importance of such therapeutics. Before we introduce peptide and protein nanostructures, we will briefly touch on the peptides and proteins that make such nanostructures and how these have been used and engineered for different purposes.

Semaglutide is a recombinant polypeptide recently marketed by Novo Nordisk with brand name Ozempic. Semaglutide is similar to endogenous human glucagon-like peptide-1 (GLP-1) and is thus an effective GLP-1 receptor agonist.[13] Since its inception, it has been advertised and used as an anti-diabetic medication due to its effect on the glucose metabolic pathway. Recent observations showed that it is also highly effective for weight loss.[14] The GLP-1 receptor activation regulates postprandial glucose levels by increasing insulin production.[15] Notably, insulin itself was one of the earliest peptide drugs to be approved for diabetes management. However, rapid elimination and clearance from the body posed a major challenge for the clinical application of GLP-1 agonists.

Another example of a peptide drug is oxytocin. Oxytocin is a peptide hormone currently used to induce and aid labor and control postpartum bleeding.[30] Oxytocin stimulates the cervix to contract and dilate to help move the baby through the birth canal. However, in recent years oxytocin has also demonstrated potential applications for the promotion of social bonding and the reduction of stress.[31] Oxytocin inhibits corticotropin-releasing factor at the hypothalamus; as such, upregulated oxytocin expression at the hypothalamus is associated with the mitigation of anxiety or stress responses.[32] While oxytocin is important for postpartum recovery for new mothers, oxytocin

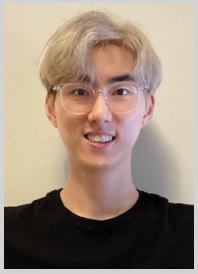

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Table 1. Peptide and protein therapeutics approved by the FDA Center for Drug Evaluation and Research (CDER) in 2023 until September[2]

#	Drug Name	Active Ingredient	Classification	Targets	Purpose	References
1	Leqembi	lecanemab-irmb	Monoclonal antibody	Amyloid beta A4 protein	Alzheimer's disease	[16]
2	Lamzede	velmanase alfa-tycv	Recombinant enzyme	N/A	Non-neurological symptoms of alpha-mannosidosis	[17]
3	Zynyz	retifanlimab-dlwr	Monoclonal antibody	PD-1	Metastatic or recurrent locally advanced Merkel cell carcinoma	[18]
4	Elfabrio	pegunigalsidase alfa-iwxj	Recombinant enzyme	Globotriaosylceramide	Fabry disease	[19]
5	Epkinly	epcoritamab-bysp	Monoclonal antibody	CD20, CD3 epsilon chain	Relapsed or refractory diffuse large B-cell lymphoma	[20]
6	Columvi	glofitamab-gxbm	Monoclonal antibody	CD20, CD3	Diffuse large B-cell lymphoma or large B-cell lymphoma	[21]
7	Rystiggo	rozanolixizumab-noli	Monoclonal antibody	FcRn large subunit p51	Generalized myasthenia gravis	[22]
8	Ngenla	somatrogon-ghla	Hormone	Growth hormone receptor	Growth failure	[23]
9	Beyfortus	nirsevimab-alip	Monoclonal antibody	Viral fusion glycoprotein F0	Respiratory syncytial virus (RSV) lower respiratory tract disease	[24]
10	Talvey	talquetamab-tgvs	Monoclonal antibody	GPRC5D, CD3	Relapsed or refractory multiple myeloma	[25]
11	Elrexfio	elranatamab-bcmm	Monoclonal antibody	TNF receptor superfamily member 17, CD3	Relapsed or refractory multiple myeloma	[26]
12	Veopoz	pozelimab-bbfg	Monoclonal antibody	Complement C5	CHAPLE disease	[27]
13	Aphexda	motixafortide	Peptide	CXCR4	Multiple myeloma	[28]
14	Pombiliti	cipaglucosidase alfa-alga	Recombinant enzyme	Cation-independent mannose-6-phosphate receptor	Late-onset Pompe disease	[29]

was also shown to play a key role in the development of the mother-infant bond.[33] Because of this, researchers are exploring the possibilities of oxytocin as a treatment for anxiety and stress-related disorders.[34]

It is important to note that both natural and synthetic peptides can be further modified. Peptide engineering can be defined as the systematic design, synthesis, modification, and functionalization of peptides to enhance their properties or introduce new functionalities for specific applications. This process may involve the alteration of the peptide's primary structure, the addition of chemical groups or moieties, or the manipulation of its physical attributes. Peptide engineering aims to optimize peptides for therapeutic, diagnostic, or other purposes by improving attributes such as stability, selectivity, efficacy, and bioavailability. Techniques in peptide engineering include amino acid substitution, conjugation of fatty acids or other molecules, and the creation of stapled peptides among others.

Antimicrobial peptides (AMPs) are an example of engineered peptides. The rise in multidrug-resistant microbes is a critical global health concern, further complicated by the enhanced resistance of microbes in biofilms, which account for approximately 75% of human chronic infections.[35] The AMPs have emerged as potential replacements for traditional antibiotics due to their unique mode of action of binding to and disrupting microbial cell membranes, reducing the likelihood of resistance development. The AMPs are essential components of the innate immune system that offer non-specific protection against various pathogens.[36] Nevertheless, AMPs are constrained by challenges such as short half-lives, protease sensitivity, non-specific hemolytic activity, and potential immunogenicity. Strategies such as amino acid isomer substitution, C-terminal amidation, and fatty acid conjugation have been explored to improve their efficacy.[36,37]

Another class of engineered peptides is stapled peptides. Peptide stapling was first introduced as a method by cross-linking side chain-to-side chain or side chain-to-end group to form preorganized stable helical conformations that increase the peptide's stability in circulation against proteolytic degradation.[38] The development of stapled peptides occupies a niche in the current repertoire of current therapeutics, where they can target protein-protein interactions that small molecules (<500 Da) or protein-based biologics (>5 kDa) are not able to target.[39] More precisely, stapled peptides have the advantages of disrupting specific protein-protein interactions with certain selectivity and specificity like protein biologics while being more resistant to protease degradation due to the nature of the staple, with the added benefit of being able to target intracellular interactions like small molecule drugs.[39] While the introduction of the staple itself is an example of peptide engineering, further tuning of the staple's design and

sequence,[40] or even incorporation into nanostructures[41] are additional engineering strategies that are being investigated.

Peptide-drug conjugates are actively being investigated for improved or new therapies. Peptide-drug conjugates are covalently conjugated to drugs and provide an additional targeting functionality. For example, conjugation of the well characterized RGD peptide, which has a high affinity for integrin receptors αVβ3 often upregulated in various tumors, directs the therapeutic payload to cancerous cells, thereby reducing off-target effects on healthy tissues.[42–44] The effectiveness of these conjugates is provided by the precise interaction between RGD epitope and integrin receptors, as well as the design of the chemical linker, which can be stable or cleavable depending on the desired drug release mechanism.[42,45] This approach marks a breakthrough in creating more targeted and efficient cancer treatments.

Protein therapeutics, including monoclonal antibodies, enzymes, and recombinant proteins, play a pivotal role in modern pharmacotherapy, particularly in treating cancers, autoimmune diseases, and genetic disorders.[46] Their high specificity and affinity for molecular targets underpin their therapeutic efficacy. Advances in biotechnology, such as recombinant DNA technology and protein engineering, have enhanced their stability, reduced immunogenicity, and improved pharmacokinetics. However, challenges such as production complexity, cost, and potential immune responses persist. Despite these challenges, protein therapeutics remain crucial in precision medicine, continually expanding their therapeutic potential and clinical utility.[47]

The field of protein engineering is equally large and there are many reviews about its potential in the literature.[48] A few examples of protein engineering methods that are currently being investigated include chemical modification of protein drugs with synthetic ligands, which can be loaded into lipid nanoparticles for targeted cancer therapeutics;[49] engineered immunoglobulins for the generation of antibody or protein transport vesicles;[50] antibody-drug conjugates for the treatment of cancers;[51] and engineered endonucleases for gene editing,[52] among others.

3. Peptide and Protein Nanostructure Formation

As peptide and protein drugs have shown promise for use in clinical settings, peptide and protein nanostructures are another way to develop innovative therapeutic materials for tackling challenges that may be difficult to address with conventional drugs. Peptide and protein nanostructures have been formulated in different structures and architectures, and characterization methods suitable for the different architectures have advanced rapidly as well. The characterization of these systems is presented in detail elsewhere.[53] Nanostructure characterization methods can be broadly separated into three categories: experimental characterization of parameters such as size and morphology, microscopy for visual confirmation, and *in vitro* and *in vivo* characterization of nanostructure stability and toxicity (Figure 1A). One constraint that all nanoparticles share is that they must be soluble and stable at physiological fluids. Taking this important point into consideration, we introduce the main methods involved in the formation of such nanostructures: electrostatic interactions, hydrophobic effect, and metal ion coordination. Here, we highlight representative studies for a variety of chemistries and present their potential in precision medicine.

3.1. Electrostatic Interactions

A therapeutic cargo has the potential to become charged under various physiological conditions, and the ability of the cargo to form electrostatic interactions can be exploited to formulate nanostructures such as polyelectrolyte, or polyion, complexes. Soluble polymers with opposite charges can form complexes in solution, releasing counterions near the polymer chains and undergoing phase separation.[54,55] The strength of the interaction among the charged moieties of the polymers determines whether such complexes will precipitate out of solution or undergo microphase separation and form coacervates. Furthermore, the addition of a neutral polymer chain can further alter the conformation of the nanostructure: in the case of neutral hydrophilic polymers like polyethylene glycol (PEG), a micellar structure arises with a charge-dense core and a neutral hydrophilic corona (Figure 1B). Other neutral hydrophobic chains have been reported as well and will be discussed below. In the case of peptide nanostructures, a defined sequence of cationic or anionic amino acids can serve as a charged polymer chain. There are many studies in the literature on the formation and characterization of polyelectrolyte complexes and complex micelles. We highlight some of the recent studies that used such structures as drug delivery vehicles for a diversity of diseases.

Electrostatic interactions can be exploited in the delivery of nucleic acids, as the phosphate backbone in every nucleic acid is naturally negatively charged. Some of the earlier works in the delivery of genetic material using polyelectrolyte complex micelles used cationic polypeptides, primarily lysine and arginine, for being positively charged at physiological pH.[56] In a recent study, microRNA inhibitors were complexed with polylysine conjugated to polyethylene glycol (PEG) to form polyelectrolyte complex micelles. Specifically, a microRNA inhibitor to miR-92a, which has been shown to promote atherosclerosis, was delivered to bind to and thus suppress pro-atherogenic endogenous miR-92a.[56] The researchers demonstrated that the complexation resulted in a monodisperse micelle population averaging 30 nm in diameter, and they further demonstrated that micelles successfully internalized *in vitro* in human aortic endothelial cells and *in vivo* in mouse endothelial cells lining the carotid artery. Interestingly, in order to specifically target inflamed vascular endothelial cells both *in vitro* and *in vivo*, a short peptide sequence targeting Vascular Cell Adhesion Molecule 1 (VCAM1), which is highly expressed in inflamed vasculatures, was conjugated to the PEG-*b*-polylysine

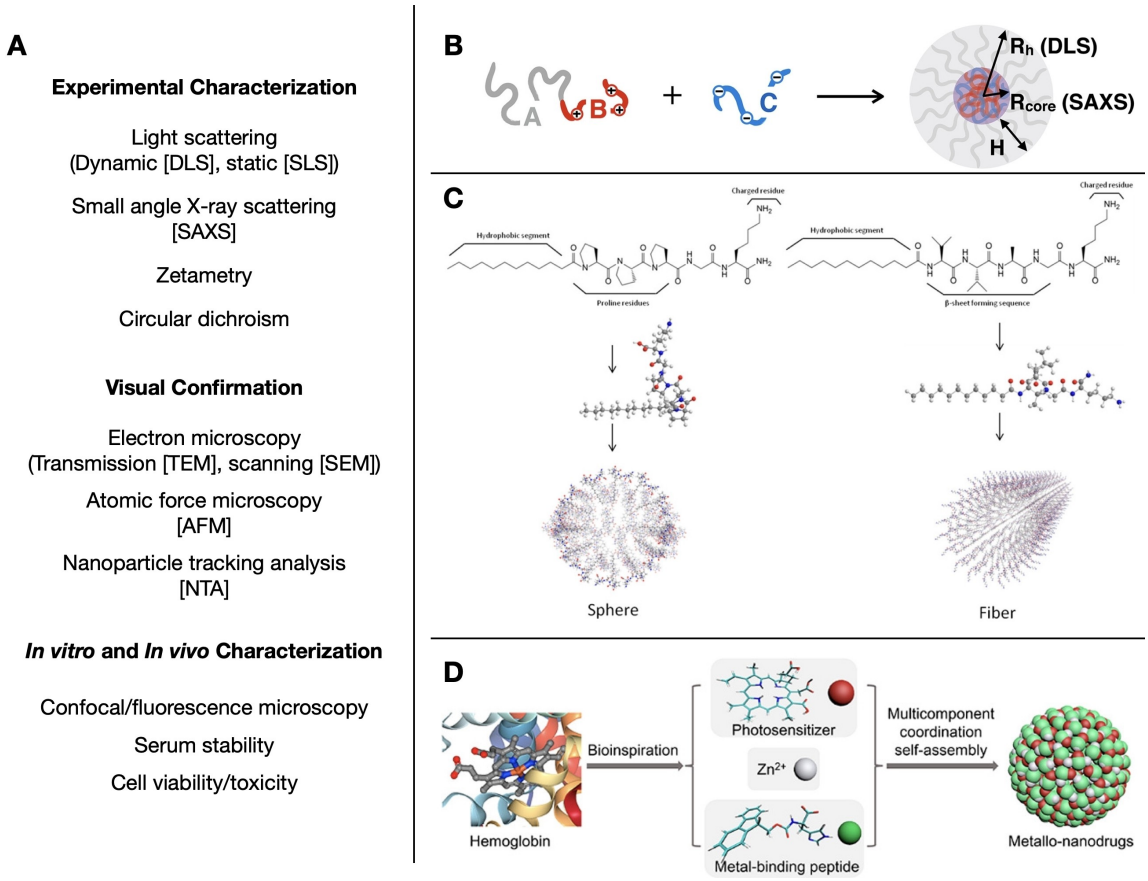

Figure 1. Examples of different methods to synthesize peptide and protein nanostructures. A) Commonly used methods for nanoparticle characterization. B) Polyelectrolyte complex micelles (PCMs) formed via electrostatic interactions. When a block copolymer of a neutral hydrophilic compound A and cationic polymer B is complexed with negatively charged nucleic acid C, a micelle with a charge-dense core and a soluble hydrophilic corona is formed (partially adapted with permission from Marras et al.[54]). B) Peptide amphiphiles with different amino acid sequences lead to different architectures. Proline residues disfavor β-sheet formation and result in spherical nanostructures, whereas Val-Val-Ala residues result in β-sheet formation and fiber-like nanostructures (partially adapted with permission from Mumcuoglu et al.[60]). C) Histidine-containing peptides and a photosensitizer both bind to Zn^{2+} ions to form metallo-nanodrugs for photodynamic therapy (partially adapted with permission from Li et al.[61]).

block copolymer. Conjugation of this targeting peptide enhanced uptake in endothelial cells and targeted inflamed vascular endothelial cells in the animal models. This strategy enabled reduced atherosclerotic burden. Similarly, poly(β-amino ester) complexes have been used to treat atherosclerosis as well.[57] After complexing with plasmid DNA encoding for interleukin-10 (IL-10), the poly(β-amino ester) complexes were further coated with PEG-VCAM1-targeting peptide conjugated to a poly-*L*-glutamic acid backbone. After validating the ability of their targeted nanoparticles to bind to VCAM1 by surface plasmon resonance with imaging, the researchers showed that their targeted nanoparticles had an order of magnitude higher affinity to VCAM1 than their nontargeted control nanoparticles. In both of these experiments, genetic material was labeled with fluorescent dyes for *in vitro* and *in vivo* imaging and tracking, which allowed for immunofluorescence staining, biodistribution characterization and fluorescence imaging tomography via *in vivo* imaging system (IVIS).

In another study, Aydinlioglu et al.[58] introduced neutral hydrophobic phenylalanine repeat units into PEG-*b*-polypeptide block copolymers made of either lysine or glutamic acid to observe the influence of increasing hydrophobicity via the amount of Phe in a nanoparticle that is still electrostatically driven to encapsulate siRNA. The researchers used multi-angle light scattering to verify the formation of spherical vesicles over micelles, and they observed that the anionic charges used to form the vesicles originated from both siRNA as well as a tripartite PEG-Glu-Phe block terpolymers. The presence of siRNA did not affect the morphology of the resulting nanoparticles as vesicles were formed when the tripartite block terpolymers of opposite charges were complexed with or without siRNA. The researchers reported that the lower hydrophilic-to-hydrophobic ratios (PEG-to-Phe weight percent ratio) formed more stable micelles as measured by less aggregation and lower polydispersity indices, even when subjected to multiple steps of extrusion on a 200 nm polycarbonate filter membrane. They attributed the differences in particle formation at different hydrophilic-to-hydrophobic ratios to previous literature showing the immiscibility of PEG to the charged components of the particle as well as increased steric hindrance at higher weight percent of PEG.

Supramolecular assemblies relying on electrostatic interactions are not limited to therapeutic nucleotides. For example,

an interleukin-1 receptor antagonist (IL-1ra) fused to a random-coil-forming pentapeptide $(VPGKG)_{72}$ motif was shown to interact with PEG or chondroitin sulfate to form spherical nanostructures.[59] In this work, IL-1ra compounds were encapsulated as a potential treatment for rheumatoid arthritis (RA), which is a chronic autoimmune disorder characterized by excessive proinflammatory cytokines leading to joint inflammation and degradation. The IL-1ra has a short half-life and necessitate frequent dosing, which often leads to lower patient compliance and higher treatment costs. To address these issues, IL-1ra was complexed non-covalently into supramolecular assemblies where the positive lysine units from the fused pentapeptide motif interacted electrostatically with either PEG with a carboxyl end group or chondroitin sulfate molecules, both of which are FDA-approved. *In vitro* studies using human fibroblast synovial cells-RA and RPMI1788 lymphocytes confirmed that these nanoparticles significantly inhibited the production of inflammatory cytokines such as PGE2 and modulated IL-1β-induced cell proliferation. When tested *in vivo*, the nano-assemblies extended the half-life of IL-1ra up to 30 hours when injected subcutaneously in a collagen-induced arthritis rat model, suggesting improved biocompatibility and bioavailability.

3.2. Assemblies Through Hydrophobic Effect

A great advantage of designing amphiphilic peptides is to improve the solubility of the hydrophobic drugs in physiological conditions through hydrophobic encapsulation. This is especially beneficial in the context of diseases where treatment options are limited and often rely on the administration of compounds with moderate cytotoxicity, as nanostructures could increase the circulation time of compounds in the blood and thus potentially reduce the dosing frequency.[62,63] Additionally, amphiphilic building blocks can facilitate the co-delivery of both hydrophobic and hydrophilic compounds. Lipid or liposomal nanoparticles and peptide amphiphiles are examples of nanoparticles that depend on hydrophobic interactions for effective cargo delivery. First, we specifically focus on hydrophobic collapse; it is worth mentioning that this phenomenon is commonly seen in peptide amphiphiles containing aliphatic amino acids. However, π-π stacking and aromatic interactions are also important contributors to hydrophobic interactions and will be discussed later.

Lipid nanoparticles and lipid-peptide conjugates, such as peptide amphiphiles, are primarily assembled via hydrophobic effect. Interestingly, peptide amphiphiles, which are hydrophilic (charged) peptides conjugated to a hydrophobic tail, can be mixed with lipid components to drive the self-assembly of multiple structures such as micelles, liposomes or multilamellar structures.[64] One example of the diverse structures possible with peptide amphiphiles was provided in an article by Mumcuoglu et al.,[65] where they synthesized two peptide amphiphiles via solid phase peptide synthesis that either favored or disrupted β-sheet formation, where a lauryl hydrocarbon chain was covalently conjugated to peptides of defined sequences. Specifically, a Val-Val-Ala sequence within the peptide favored β-sheet formation, whereas a substitution to three prolines disfavored such conformation (Figure 1C). These peptides were conjugated to additional residues: a lysine residue was incorporated to complex with antisense oligonucleotides (ASOs), and a serine residue was used to conjugate glucose to serve as a targeting moiety for cancer. When complexed with ASOs, the β-sheet favoring peptide amphiphiles formed nanofibrillar structures as seen under TEM and circular dichroism spectra, whereas their counterparts formed spherical nanostructures. Interestingly, when visualized via confocal microscopy, although nanospheres entered the cells more quickly, the nanofibers delivered more amounts of labeled ASOs as measured by fluorescence intensity. Additionally, the different architectures entered the cells via different mechanisms: the authors showed that nanospheres seemed to passively diffuse into and out of the cells, whereas nanofibers entered the cells primarily by diverse endocytosis pathways as shown by preventing specific endocytosis pathways via selective inhibitors. Then, MCF-7 lung adenocarcinoma cells were used to determine whether the additional glucose moiety could take advantage of the Warburg effect in cancer cells, which refers to the increased rate of glycolysis and glucose metabolism of cancer cells. By administering two different glucose transporter inhibitors, the researchers showed that uptake of the glucose-conjugated, β-sheet-favoring peptide amphiphile nanostructure was significantly decreased as compared to its non-inhibited controls.

In addition to the direct interaction of peptide amphiphiles with their cargo prior to delivery, these nanostructures can further be imbedded to hydrogels or other substrates. For example, doxorubicin, a hydrophobic chemotherapeutic agent, could be encapsulated in a peptide amphiphile hydrogel as an injectable local delivery system.[62] Doxorubicin is highly cytotoxic when administered intravenously and thus necessitates an efficient delivery platform that can reduce toxicity while maintaining or enhancing therapeutic effect. Peptide amphiphiles of different charges were synthesized consisting of a hydrocarbon tail, a β-sheet forming motif and either cationic (lysine) or anionic (glutamic acid) amino acids, which endowed them the capability to self-assemble into hydrogels via both electrostatic and hydrophobic interactions in water at pH 7.4. Charge neutralization occurred at a molar ratio of 3 cationic : 4 anionic peptide amphiphiles at 0.5% (w/v) concentration, at which point they formed nanofibers as observed by TEM and verified by CD and Fourier Transform Infrared Spectroscopy (FTIR). Rheology was additionally performed to show that despite the gel forming through noncovalent interactions, the gel possessed the necessary mechanical stability for local soft tissue applications, and the gels were further incubated with proteases to show that they are indeed biodegradable but could still last multiple weeks. The gel remained stable and showed stable release profiles for 156 hours, and the authors proposed that drug availability could improve when injected locally due to the biodegradability of the gel. When 4T1 breast cancer cells were cultured in the presence of hydrogel, the hydrogel with no drug did not exhibit cytotoxicity, but both

Dox (doxorubicin treated directly on culture media) and Dox/hydrogel conditions exhibited similar decreases in cellular viability by 24 hours. Nevertheless, growth inhibition was sustained in the Dox/hydrogel condition as compared to Dox alone, suggesting that the hydrogel enhanced doxorubicin bioavailability and activity.

Hydrogels can also be stabilized by aromatic interactions, π-π stacking. For instance, diphenylalanine (FF) is a well-known peptide sequence for its ability to self-assemble into nanotubes.[66] In one example, a double network hydrogel suitable for drug delivery applications was synthesized by chemically cross-linking a Phe-Phe-Lys peptide to polyethylene glycol diacrylate polymers via Michael addition.[67] Because of the π-π stacking interactions of FF, a second layer of cross-linked network resulted in a hybrid double network hydrogel. Preliminary data suggested that the hydrogel maintained favorable characteristics (mechanical properties, slow degradation) even in blood and *in vitro* cell culture, supporting the potential use in drug delivery and other applications such as tissue engineering.

In a different example, peptide amphiphiles composed of a 12-, 13-, and 14-carbon alkyl tail conjugated to F_2E_2 (Phe-Phe-Glu-Glu) were studied for their potential to form hydrogels.[68] Intriguingly, a $[C_{12}]UF_2E_2$ peptide amphiphile was also designed, where a urea group (U) was placed between the lipid and the peptide portion to observe the effects of additional hydrogen bonding capability. Most of the peptide amphiphiles formed nanoribbons or nanobelts of different widths, attributed to both the hydrophobic collapse of alkyl tails and π-stacking. When the peptide amphiphiles were allowed to gel by the addition of calcium ions to form crosslinks with the negatively charged Glu residues, only the urea-containing version formed aligned bundle-like structures in the hydrogel. These urea-containing structures had the best stability against mechanistic stress and self-healing ability while possessing good stability for long-time cell culturing. This observed difference was attributed to an increased number of hydrogen bonds and the urea-π interactions with the Phe side chain.

3.3. Self-Assembly via Metal Ion Coordination

Peptides are also capable of self-assembling into supramolecular structures via metal ion coordination. Examples of peptide-metal coordination include coordination through terminal amino group and carbonyl, terminal amino group and terminal histidine imidazole, and terminal amino group and terminal cysteine thiolate.[69] It is important to note that noncovalent interactions such as electrostatic interactions and hydrophobic effect also play important roles in stabilizing such structures. Metal-peptide assemblies include macrocycles, catenanes, helicates, and even 2D and 3D metal-peptide layers and frameworks.[70] Hexahistidine, for example, has been shown to dimerize in the presence of transition metal ions. Interestingly, when conjugated to an oligostyrene hydrophobic tail, the peptides self-assembled into different structures depending on the divalent metal ion present. In the presence of Mn(II), they formed multilamellar vesicles; in the presence of Co(II) and Cu(II) they formed aggregated micelles, and in the presence of Ni(II) and Cd(II), they formed micelles, reinforcing the idea that a single material can have a diverse set of responses to different metal ions.[71]

Such characteristics have been exploited in multiple applications. For instance, metal ions have been incorporated into supramolecular gels to self-assemble into hydrogels capable of loading therapeutics. A hexapeptide composed of three glutamic acid residues and three phenylalanine residues was found to form hydrogels in the presence of zinc ions but not with other divalent metal ions found in the blood or tissue, which made it a suitable vehicle for an injectable prostrate-targeted drug delivery in which there is a high concentration of zinc ions.[69,72] When loaded with anti-cancer drug docetaxel, the system showed high anti-cancer efficacy against prostate cancer cells while exhibiting no cytotoxicity in normal liver cells. In another example, histidine-containing short peptides, photosensitizes and metal ions resulted in yet another injectable metallo-nanodrug for photodynamic therapy.[61] Two histidine-containing peptides, fluorenylmethoxycarbonyl(Fmoc)-L-histidine and N-benzyloxycarbonyl-L-histidine-L-phenylalanine, were able to form spherical, solid nanoparticles with average diameters of around 75 nm in the presence of Zn^{2+}, and the formation of nanoparticles did not significantly differ when a photosensitizer was also included in the system (Figure 1D). Multicomponent and cooperative coordination of the photosensitizer and the short peptides resulted in similarly sized spherical nanoparticles. Because of the nearly covalent characteristics of metal coordination interactions, the resulting nanoparticles were highly stable under normal physiological conditions and enhanced the blood circulation time of the photosensitizer as compared to when the photosensitizer was uncomplexed.

4. Therapeutics Loading into Nanostructures

Peptide nanostructures can be formulated and modified in various ways, and the breadth of choice is similarly large when it comes to what to deliver. Drug compounds (such as chemotherapeutics) and gene therapy molecules (different forms of DNA and RNA) are a few examples from this continuously expanding list. The delivery method, synthesis, and formulation approaches are highly dependent on the chemistry of the cargo being delivered. The methods for conjugating peptides, proteins or other moieties to nanostructures have been reviewed extensively elsewhere.[73] Therefore, we provide a few examples that have reported interesting approaches to loading the cargo and protecting it from degradation until delivered to the target cell.

4.1. Loading of Therapeutic Compounds

Multiple studies have investigated the efficacy of peptide nanostructures for the treatment of cancer. Many compounds

used to treat cancer are hydrophobic and thus are loaded or encapsulated into nanostructures via hydrophobic collapse. Therefore, lipid-containing nanostructures or supramolecular peptide amphiphile assemblies are commonly used. Tumor-targeting liposomal nanoparticles conjugated to anti-EGFR antibodies have been reported to successfully treat non-small cell lung cancer.[74] One big challenge in cancer is the development of resistance to specific chemotherapeutics, necessitating yet another mode of intervention and treatment. The researchers noted that many patients exhibiting an EGFR mutation become resistant to EGFR tyrosine kinase inhibitors (TKI) through a process in which epithelial cells transition into a different cell type. However, administration of 1,25-Dihydroxyvitamin D3 (1,25D3) has been shown to promote epithelial differentiation and thus could prove to be effective against EGFR TKI resistance. In combination with a selective hydroxylase inhibitor (CTA091) to prevent the inactivation of 1,25D3, the researchers formulated a liposomal nanoparticle decorated with anti-EGFR antibodies as EGF receptors are highly upregulated in EGFR mutant non-small cell lung cancer. The liposomal nanoparticles were formulated by first mixing both 1,25D3 and CTA091 with the lipid components, and then inserting anti-EGFR antibodies conjugated to DSPE lipid-PEG into the mix. After establishing an *in vitro* EGFR TKI resistant cell line, the liposomal nanoparticle was first formulated with labeled oligonucleotides and shown to be internalized by EGFR TKI resistant cells more readily than the non-targeted liposomal nanoparticles or free labeled nucleotides as determined by flow cytometry and confocal microscopy. Because this was a co-delivery system, the dosage of each compound was determined sequentially and the effectiveness was tested *in vitro*, where the researchers showed promising qPCR data that 1,25D3 increased the expression of the hydroxylase while also increasing *CDH1*, a known epithelial marker. They then further determined that the transcriptional changes are also reflected in protein expression and ultimately resulted in less tumor cell colony formation, an indication of growth inhibition.

In another study, a different peptide sequence was used to target EGFR for glioblastoma.[63] D-AE, the enantiomer of the L-AE peptide, which was discovered through a mixture-based synthetic combinatorial library to bind specifically to EGFR and its mutant form EGFRvIII, was conjugated to PEG-polylactic acid micelles encapsulating paclitaxel (Figure 2A). They reported that L-AE, although specific for the EGF receptors, was proteolytically unstable and resulted in impaired targeting efficiency, while D-AE was more proteolytically stable. The peptides were conjugated to the PEG-polylactic acid block copolymer via thiol-maleimide click chemistry and successful conjugation was verified by ^{1}H NMR. The block copolymer was designed to function as a delivery vehicle able to encapsulate the hydrophobic drug paclitaxel; micelles were formed via thin film hydration, where the peptides and paclitaxel were initially

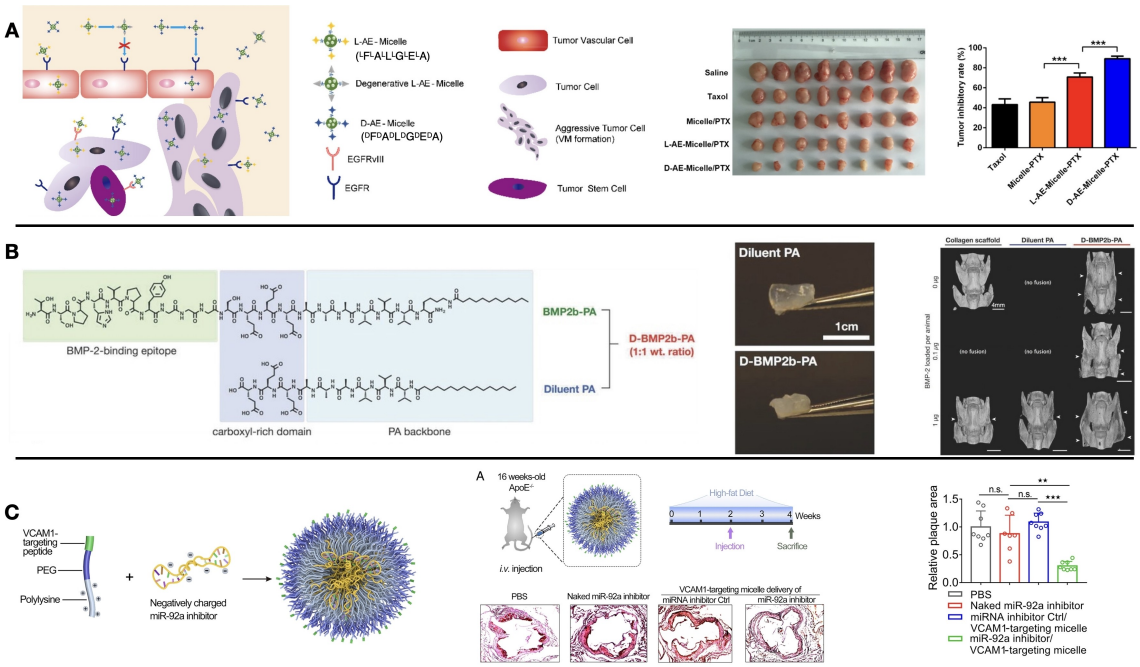

Figure 2. Examples of therapeutics that can be encapsulated or delivered using peptide nanostructures. A) Micelles decorated with enantiomers of EGFR-targeting peptides were formulated to encapsulate paclitaxel (PTX) for the treatment of glioblastoma. Specifically, D-AE peptides showed the greatest reduction of tumor size and a greater rate of tumor inhibition in mice bearing subcutaneous U87 tumors (partially adapted with permission from Mao et al.[63]). B) Peptide amphiphiles for the supramolecular assembly of nanofibers were reported for the treatment of pseudarthrosis. A peptide amphiphile (PA) molecule conjugated to a BMP-2-binding epitope peptide was diluted with non-conjugated PAs at a 1 : 1 weight ratio to form a nanofiber gel that retained BMP-2 growth factor for longer periods of time and resulted in successful bone fusion (partially adapted with permission from Lee et al.[80]). C) Polyelectrolyte complex micelles were formed to encapsulate a short therapeutic nucleotide (microRNA-92a) for the treatment of atherosclerosis. The micelles were decorated with a Vascular Cell Adhesion Molecule-1 (VCAM-1)-targeting peptide for targeted delivery to inflamed endothelial cells. In a partial carotid ligation model of accelerated atherosclerosis, the targeting micelles exhibited higher therapeutic effect as noted by lower plaque area from histological analyses (partially adapted with permission from Zhou et al.[56]).

rotary evaporated to form a thin film layer and were then reconstituted in physiological saline and filtered to remove unencapsulated drug. The researchers reported that the micelles, when formed with labeled peptides, localized primarily in tumor cells and the vasculature *in vitro* and showed higher *in vivo* localization within tumors derived from human glioblastoma cells injected subcutaneously in mice. Because the localization of the micelles in the desired target tissues did not guarantee the cargo to be released at the target site, they further examined and confirmed that the cargo was also released at the target site via *in vitro* models of the blood-brain tumor barrier by observing the potential of the micelle to penetrate a tumor spheroid in a co-culture system and again *in vivo* within the tumors.

The application of peptide nanostructures for the treatment of cancers is numerous: camptothecin (an anti-cancer molecule) or its derivatives have been loaded in various peptide amphiphile structures successfully to increase its solubility and efficiency in various cancers;[75,76] pillararene-based supra-amphiphiles, which self-assemble into nanofibers or nanoparticles depending on temperature and thus allowing for additional thermo-responsiveness,[77] and peptide vesicles (peptidesomes) composed primarily of cyclic and amphiphilic peptides[3] or protein nanocages decorated with RGD peptides[78] were used to encapsulate photodynamic therapy (PDT) compounds. Interestingly, most photosensitizers are hydrophobic, which makes peptide amphiphiles great candidates for efficient delivery and uptake. It is important to note that most self-assembling peptides used in conjunction with antitumor drugs are still susceptible to degradation by endogenous proteases, potentially resulting in premature drug release. This challenge has been partly addressed by introducing D-amino acids, which enhance biostability against proteases.[63,76,79]

Applications of peptide nanostructures outside of cancer exist as well. For instance, peptides have been used for the formation of supramolecular nanofibers in promoting osteogenesis as a possible treatment to pseudarthrosis, the nonunion of bones or improper bone healing after fractures or spine fusion surgeries.[80] Conventionally, recombinant human bone morphogenetic protein-2 (BMP-2) with collagen has been used for enhanced bone formation, although its use necessitates high doses, leading to potential complications. Subsequently, peptide amphiphiles containing a BMP-2 binding sequence were designed as optimal scaffolds to reduce BMP-2 dosage, which not only enhanced the BMP-2-induced osteoblast differentiation *in vitro* but also showed prolonged retention of the growth factor when formulated into a gel (Figure 2B). In practical tests using a rat spinal fusion model, this nanofiber gel achieved a 100% fusion rate when loaded with a BMP-2 dose that was ten times lower than what was typically needed with a collagen sponge. Remarkably, even without the addition of exogenous BMP-2, a 42% spinal fusion rate was observed, suggesting that the nanofibers appeared to mimic some features of natural polysaccharides, enhancing BMP-2's osteoblastic activity. Furthermore, the release rates of BMP-2 from these gels were slower than those from collagen sponges, offering the potential for more sustained therapeutic effects.

In another example, lipoproteins decorated with matrix metalloproteinase-9 (MMP-9) activated cell-penetrating peptides (which we discuss in more detail later) were reported for use in traumatic brain injury.[81] Traumatic brain injury, or more specifically secondary cerebral injuries following traumatic brain injury are characterized by mitochondrial dysfunction, calcium imbalance, and a plethora of neuroinflammatory responses, which compound the primary mechanical damage, often leading to irreversible neuronal loss. Therefore, research into the targeted delivery of neuroprotective agents to the affected sites in the brain to confer maximum therapeutic benefits while minimizing systemic side effects is of great importance. Consequently, a targeted nanocarrier system using reconstituted lipoproteins loaded with Cyclosporin A (CsA), an immunosuppressive drug with neuroprotective properties, was developed to modulate the mitochondrial permeability transition pore and decrease reactive oxygen species production.[81] Because MMP-9 is overexpressed at traumatic brain injury lesion sites, an MMP-9 sensitive peptide shell was integrated to the nanocarrier. Utilizing a controlled cortical impact mouse model, the study demonstrated the nanoparticles' enhanced targeting ability and achieved significantly higher accumulation at the lesion sites, as compared to conventional CsA delivery methods. Moreover, pharmacokinetic analysis revealed superior bioavailability of CsA when delivered via these MMP-9-sensitive lipoproteins. The nanoparticles not only improved mitochondrial function but also attenuated post-traumatic brain injury neuropathological changes at a fraction of the CsA dose commonly required.

4.2. Loading of Therapeutic Nucleotides

As mentioned above, peptide nanostructures have been developed for the delivery of therapeutic nucleotides.[82,83] For instance, a polyelectrolyte complex micelle encapsulating miR-92a inhibitors were used for the treatment of atherosclerosis and thus the possibility of delivering nucleotides as therapeutics[56] (Figure 2C). Specifically, the delivery of miR-92a inhibitors resulted in a reduction in plaque area, and despite the fact that the unencapsulated miR-92a inhibitor showed some therapeutic effect, its effect was greatly enhanced when a delivery vehicle with an additional targeting peptide was employed. Because all nucleic acids are connected by phosphodiester bonds, they are naturally negatively charged and thus can form electrostatic interactions with cationic compounds..[82,83] The DNA and RNA therapeutics are crucial components for gene therapy, but a big roadblock on their systemic delivery is their instability in biological, physiological fluids, and thus these most often require delivery vehicles. For example, RALA, a 30 amino acid amphipathic fusogenic peptide sequence of WEARLARALARALARHLARALARALRACEA, has been used to synthesize gene delivery vehicles.[82] Its unique sequence, containing multiple arginine and leucine repeats, facilitates condensation of nucleic acids and allows spontaneous production of nanoparticles. For example, McCrudden et al. designed a study to investigate the complexation,

characterization, and application of RALA/plasmid DNA encoding for a reporter gene or iNOS in prostate cancer cells and *in vivo* studies.[82] Nitric oxide has been identified to play a concentration-dependent role in cancer, with superphysiological concentrations promoting anti-cancer effects. Nanoparticles formulated using cationic amphipathic peptide RALA in combination with plasmid DNA exhibited a positive surface charge of approximately +30 mV, suggesting a stable colloidal system. Dynamic light scattering techniques further validated these findings, indicating a uniform nanoparticle size distribution with an average diameter around 100 nm. Importantly, these nanoparticles demonstrated a remarkable ability to transfect cells and facilitate gene expression, as assessed through both qPCR and fluorescence microscopy. Additionally, *in vivo* studies utilizing immunocompetent murine models confirmed that the administration of these RALA/plasmid DNA nanoparticles did not elicit an undesirable immune response, an essential criterion for translational success.

A different study demonstrated the ability to utilize a peptide-based nanoparticle system called p5RHH designed for targeted and efficient delivery of small-interfering RNA (siRNA) against KRAS.[84] The KRAS is an oncogene that is implicated in approximately 95% of pancreatic ductal adenocarcinoma cases, which remain one of the most intractable and fatal cancers with a 5-year survival rate of only 8%. Despite this, KRAS has been long considered 'undruggable' due to its unfavorable biochemical properties. To overcome this challenge, a multi-modal approach was employed for targeted gene silencing in KRAS-driven malignancies using siRNA . The nanoparticles were meticulously engineered to a size of 55 nm and a positive charge of +12 mV. These attributes were strategically selected to exploit the enhanced permeability and retention (EPR) effect commonly observed in tumor vasculature, and to facilitate interaction with the net negative charge commonly found on the surface of cancer cells. *In vitro* studies demonstrated high uptake levels using fluorescently labeled nanoparticles, reaching an average of 94.3% across seven pancreatic and colorectal cancer cell lines. Subsequent KRAS-siRNA nanoparticle treatment led to significant reductions in KRAS RNA and protein expression, as well as in downstream pERK levels in different murine cancer cell lines. *In vivo* experiments in murine tumor xenograft models and in a genetically engineered mouse model of spontaneous pancreatic ductal adenocarcinoma revealed significant accumulation of nanoparticles within tumors, liver, and kidneys, 24 hours post-intravenous injection.

5. Facilitating Therapeutics Delivery

Peptide and protein nanostructures can enhance the stability, solubility, and bioavailability of the therapeutic agents, while the functional moieties on their surface enable targeted recognition and binding to specific cells or tissues. By combining the unique properties of peptides, proteins and nanostructures, drug targeting strategies can achieve enhanced therapeutic efficacy, reduced off-target effects and improved patient outcomes in a wide range of diseases.

5.1. Surface Functionalization

Through the incorporation of certain bioactive markers and molecules, peptide nanostructures can be functionalized for a wide range of therapeutic applications. One such method is the conjugation of polyethylene glycol, commonly referred to as PEGylation. The PEGylated peptide-based materials have demonstrated improved pharmacokinetic profiles during biopharmaceutical delivery.[82,83,85] Methods of site-specific PEGylation have been successfully approached from both a chemical and an enzymatic point of view.[86] The PEGylating therapeutic peptides based on endogenously expressed proteins can reduce the chance of an immunogenic reaction in clinical settings. These biochemical modifications increase the size of the compound, leading to reduced clearance and thus an increased half-life in the body.[87] Even then, the circulation lifetime of some peptide-based drugs may be short, where rapid degradation and excretion of these drugs may require more frequent dosing.[88] A recent study demonstrated that PEGylation of proteins interferes with secondary structure formation of hydrophobic regions near the cell membrane, and that this effect became exacerbated when attaching PEG molecules of higher molecular weight compared to those with lower molecular weight PEG.[89] Grafting multiple copies of amphiphilic peptides to heavier PEG molecules increased delivery efficiency but were more cytotoxic than the native peptide alone.[89]

Physiological lipidation is another method of post-translational modification where fatty acids are covalently linked to peptides and proteins.[90] Acylating peptides with longer-chain lipids has been demonstrated to extend the duration of their biological action, either by binding with carrier proteins like serum albumin or through self-aggregation.[91] Recently, the synthesis and application of lipidated proteins has focused on the study of reversible lipidation techniques.[92] Reversible lipidation takes advantage of the chemical properties of fatty acid chains, enabling peptides to switch between hydrophobic and hydrophilic conformations.[93] Thus, depending on the surrounding physiological environment, these "smart" peptide drugs can take on the structure allowing for the most efficient delivery and biological activity at their injection site. For example, it has been long established that fatty monoacids are more hydrophobic than fatty diacids, which contain an ω-carboxylic group that increases their solubility in biological environments.[94] Therefore, modulating peptides with more fatty monoacids results in higher protein-cell membrane associations and facilitated endocytosis.[95] However, fatty diacids were found to offer much stronger albumin affinity, and thus longer half-lives when bound to albumin carrier proteins.[96] Thus, different acylation methods may be more appropriate depending on the cargo of interest.

Connecting fatty acids to a peptide backbone requires the inclusion of a spacer element and a linker element. The linker element refers to the short peptide segment connecting the fatty acid to the spacer or onto the peptide backbone itself. The linker element can be easily substituted or even omitted in fatty monoacid functionalization; however, its presence is essential

for functionalization with fatty diacids. For instance, it was found that incorporating L-γGlu into the peptide as a linker provided the greatest albumin affinity, and thus the longest duration of biological activity when functionalizing the peptide with fatty diacids.[97] The spacer element, which is another short peptide sequence between the linker and peptide backbone, may affect the binding affinity for the target receptor.[97] The use of longer spacers attenuates the negative effect on receptor binding.[96] Shorter spacers can also sterically shield the peptide from *in vivo* degradation.[98] In a few cases, functionalizing a protein with a fatty acid without the use of a linker or spacer element can still markedly increase its half-life.[99]

It is also possible to modify peptide nanostructure surfaces with other functional peptide molecules. Cell-penetrating peptides (CPPs) have emerged as promising treatments for infections, as well as delivery methods for vaccines, drugs, and other therapeutic nanostructures.[100,101] The CPPs are short peptides, with lengths of 5 to 30 amino acid residues, that can translocate into cells through energy-independent pathways without disrupting plasma membranes. They typically consist of positively charged amino acids, though they may also simply contain alternating polar and nonpolar amino acid sequences.[100] CPPs have wide structural diversity, allowing for many different modes and levels of uptake. Endocytosis[102] and direct translocation[103] through the cellular membrane are the main routes for cells to uptake CPPs. Historically, the Tat protein derived from the HIV-1 retrovirus has been one of the most studied CPP since its potential was first discovered in 1997.[104] Exogenous Tat protein can translocate through cell membranes and reach the nucleus to activate the viral genome for HIV-1 replication; a specific region of amino acids on the Tat protein is responsible for this translocation activity. Since then, Tat-derived proteins have been covalently bound to many types of cargo, such as antisense oligonucleotides for P-glycoprotein inhibition[105] and quantum dots for cellular probing research.[106]

Generally, CPPs are related by high sequence identity and common structural features, although they typically do not have sequence homology. A CPP is cationic if it contains a positively charged amino acid sequence that is necessary for cellular uptake, and that does not form an amphiphilic helix in its tertiary conformation. Studies suggest that at least five positive amino acid residues are needed for efficient uptake of several cationic CPPs.[86] Some cationic CPPs, called nuclear localization sequences (NLSs) are short peptides containing lysine, arginine, or proline residues. Due to their short lengths, they rarely have enough positive charges to be efficient CPPs on their own; however, they can be covalently attached to hydrophobic peptides to create an amphipathic CPP with an improved uptake profile.[107] Amphipathic CPPs can be further classified into primary and secondary amphipathic CPPs. Primary amphipathic CPPs (paCPPs) typically contain 20 or more amino acids, with sequential hydrophobic and hydrophilic residues along their primary structure.[108] Secondary amphipathic CPPs (saCPPs) often contain a smaller number of amino acids compared with primary amphipathic CPPs. Most CPPs form α-helices, but saCPPs can also form β-sheets upon interaction with phospholipid membranes while retaining penetrative activity.[108]

A recent application of CPPs has been in the treatment of conditions of the central nervous system, such as neurodegenerative diseases (Parkinson's, Alzheimer's, etc), stroke, and brain cancers.[109] Few drugs have proven successful in treating diseases of the CNS; it is difficult for drugs to be transported across the blood-brain barrier, which significantly limits the therapeutic efficacy of such treatments.[110] The CPP functionalization of a small molecule drug or a nanoparticle drug carrier can improve a drug's ability to cross the blood-brain barrier membrane and assist in more effective drug delivery (Figure 3A).

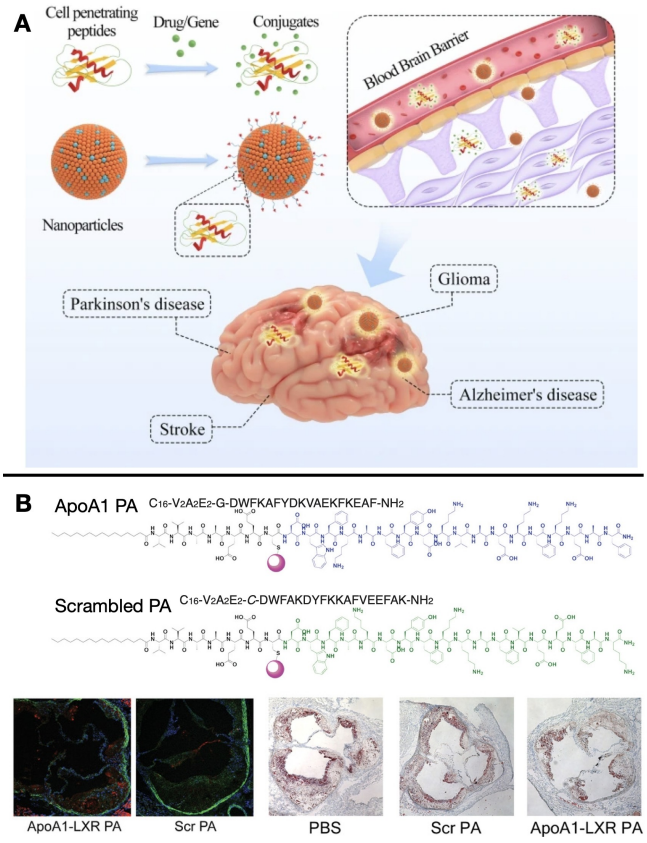

Figure 3. Examples of peptide nanostructures with cell-penetrating or targeting capabilities. A) CPPs can be conjugated to genes or small molecule drugs or used to functionalize nanoparticles. These CPP-functionalized structures can then noon-disruptively enter membranes. The above example shows how CPP functionalization allows nanoparticles or small molecule drugs to cross the blood-brain barrier and treat glioma, stroke, and other neurodegenerative conditions (partially adapted with permission from Zhang et al.[118]). B) Peptide amphiphile nanofibers were synthesized for the treatment of atherosclerosis. An ApoA1 mimetic peptide was conjugated to peptide amphiphiles for the targeted therapy of atherosclerotic plaques, and enhanced uptake using the targeting peptide was verified by fluorescence microscopy. When combined with liver X receptor agonist, the platform resulted in decreased lesions as seen via a marked decrease of Oil Red O in histological sections of aortic roots (partially adapted with permission from Mansukhani et al.[117]).

5.2. Targeting Strategies

Targeting modalities can enhance the specificity and effectiveness of nanostructures.[56,57] Targeting strategies are a major area of research, especially where systemic administration of therapeutics is required due to a lack of local delivery options. Interestingly, peptides that show specific affinity to certain receptors, tissues, or organs have already been developed and used on the benchtop and in the clinic. These targeting or homing peptides are frequently identified by combinatorial phage display techniques where bacteriophages that encode for a high-affinity peptide binding a target protein are sequenced and cloned.[111] Although this platform is conventionally used when designing ligands for a known specific target for diagnostic or therapeutic purposes,[112] peptides identified in this manner can also be conjugated to nanostructures for site-specific delivery with reduced off-target effects. As ligands to specific receptors or adhesion molecules to specific cell types, these peptides can facilitate receptor-mediated endocytosis which leads to accumulation of the therapeutic at a target site.[113]

Unsurprisingly, tumor-homing peptides are of great interest for the delivery of anti-tumor drugs and chemotherapeutics in a site- or organ-specific manner to reduce cytotoxicity in non-tumorigenic tissues (Table 2). For instance, cyclic RGD (cRGD) peptides and its derivatives like c(RGDfK) and E-[c(RGDfK)2] have garnered attention due to their high selectivity to integrin receptors, commonly overexpressed in cancer cells. These peptides, especially E-[c(RGDfK)2], offer potential advantages over other ligands due to their selectivity, stability, and lower immunogenicity.[114] In a recent paper, gambogic acid (GA), a potent anticancer compound that has shown promise in inhibiting proliferation, inducing apoptosis, and reversing multidrug resistance in breast, lung, prostate, and pancreatic cancers, was incorporated into nanostructured lipid carriers (NLCs) that allowed for increased drug loading capacity and modulated drug release. Although tumor targeting therapies can exploit the enhanced permeability and retention (EPR) effect of tumor tissues, purely passive targeting is often insufficient to deliver an effective therapeutic concentration. Therefore, both monomeric and dimeric cRGD peptides, which are renowned ligands for tumor targeting particularly via the integrin αvβ3 receptor, were functionalized onto the nanoparticle. Integrin αvβ3 has heightened expression in many cancer lines. The conjugation was accomplished using DSPE-PEG2000-COOH as a linker, chosen for its optimal cellular uptake features in cancer cells. Furthermore, dimeric cRGD-decorated GA-NLCs demonstrated enhanced uptake, superior anti-proliferative effects, and higher biocompatibility.

In a different study, a novel taxol–CPP conjugate was successfully synthesized, encompassing three integral components: the hydrophobic drug taxol and a tumor-specific cell-penetrating peptide RLYMRYYSPTTRRYG connected by a succinic acid alongside tripeptide EEG as linkers.[115] It is interesting to note that anticancer drug taxol itself was reported to have self-assembly properties. Intriguingly, when dissolved in aqueous mediums, this conjugate displayed a propensity to form homogeneous nanospheres, with a diameter of approximately 130 nm as deduced from TEM and DLS measurements. This self-assembly property was primarily attributed to the inherent characteristic of taxol to form hydrophobic interactions, as the standalone EEGRLYMRYYSPTTRRYG peptide lacked self-assembly potential. The *in vitro* cytotoxicity evaluations conducted on human hepatocellular carcinoma (HepG2) cells revealed that taxol–CPP nanospheres maintained a comparable therapeutic efficacy to free taxol. In a promising advancement, these nanospheres were also demonstrated to efficiently co-deliver another anticancer agent, doxorubicin, to hepatocellular carcinoma cells, with fluorescence microscopy confirming intracellular release and distribution of doxorubicin.

In another study, lipopeptides consisting of E_4 [$(EIAALEK)_4$] or K_4 [$(KIAALKE)_4$] peptides conjugated to cholesterol via a PEG linker (referred to as CPE_3 or CPK_3 based on the peptide sequence) were incorporated into liposomes to study peptide-sequence specific membrane fusion.[116] These lipopeptides were inspired by SNARE protein subunits used by neurons during exocytosis where complementary protein subunits on

Table 2. Examples of targeting peptide-drug conjugates of drugs that are FDA-approved or currently in clinical trial.

Target	Peptide Family	Encapsulated Drug
αVβ3 integrin	RGD and derivatives	Camptothecin[120]
		Sunitinib[121]
		Paclitaxel[44,122]
		Doxorubicin[123]
		Mirdametinib[124]
		Daunorubicin[125]
SSTR (somatostatin receptors)	Somatostatin	Lutetium Lu 177 dotatate[126,127]
		Nendratareotide uzatansine[128]
		Edotreotide yttrium Y-90[127,129]
		Camptothecin[130]
EphA2 receptor	EphrinA ligands and derivatives	Gemcitabine[131]
		Paclitaxel[132]
		Maytansine derivative[133]
GnRH-Receptors	GnRH (gonadotropin hormone-releasing hormone)	Daunorubicin[134]
		Gemcitabine[135]
KRT1 (keratin)	WxEAAYQrFL (Breast cancer targeting peptide)	Doxorubicin[136]
HER2	Anti-HER2 peptide mimetic	Doxorubicin[137]
LRP-1	Angiopep-2	Paclitaxel[138]
Transferrin receptor (TfR)	HAIYPRH	Doxorubicin[139]
	CAHLHNRS	Doxorubicin[140]
CD13	NGR tripeptide	Daunorubicin[141]

opposing membranes are brought to proximity to induce lipid mixing and content transfer via coiled-coil formation and interaction; by incorporating this concept into drug delivery, the authors were able to show that when HeLa cells are preincubated with CPK_4 liposomes and subsequently exposed to doxorubicin-loaded CPE_4 liposomes, doxorubicin was successfully transferred and resulted in cytotoxicity. Control experiments supported the idea that cytotoxicity occurs only through lipid mixing of the two liposomes. This example shows that peptides used for targeting do not always need to target a membrane receptor, further broadening the applicability and field of discovery.

Targeting peptides for diseases other than cancer are also being actively investigated. For example, capitalizing on the cholesterol-efflux property of Apolipoprotein A1 (ApoA1), an 18 amino acid ApoA1 mimetic peptide 4F was synthesized and attached to peptide amphiphiles that formed supramolecular nanofibers for the targeted treatment of atherosclerosis[117] (Figure 3B). Specifically, ApoA1 is found in high-density lipoprotein cholesterol, and is capable of promoting cholesterol efflux from atherosclerotic plaques. Therefore, ApoA1 and 4F were considered suitable targeting peptides for their ability to bind to oxidized lipids, specifically as atherosclerotic severity correlates with the presence of oxidized lipids. These nanofibers were designed to assemble with liver X receptor (LXR) agonist GW3965, which is used in the clinic for its cholesterol-regulating properties but causes liver toxicity when administered systemically. In the *Ldlr* knockout mouse model where mice develop atherosclerosis when fed with a high-fat "western" diet over multiple weeks, ApoA1-LXR PAs successfully reduced atherosclerotic burden, and while LXR and scrambled (non-targeting) PA treatments also reduced plaque formation, only ApoA1-LXR PAs demonstrated low liver toxicity profiles, suggesting that the nanostructure is necessary to deliver LXR in a highly targeted fashion.

The flexibility in peptide synthesis also allows for dual-domain peptides. For instance, this concept could be exploited for the design and synthesis of mitochondria-targeting nanostructures.[119] Mitochondrial DNA (mtDNA) is more susceptible to mutations than nuclear DNA, making it a critical target for many diseases ranging from heart failure to neurodegenerative disorders. Thus, rescuing mitochondrial function by specific and targeted delivery of functional DNA holds great therapeutic potential. Chuah et al. introduced two rationally designed mitochondria-targeting peptides: a 12-residue sequence from yeast cytochrome c oxidase subunit IV (Cytcox) and a 32-residue sequence from human ornithine transcarbamylase (OTC). In addition to these two peptides, Cytcox and OTC peptides were fused with lysine-histidine (KH) sequences (Cytcox-KH and OTC-KH, respectively) for a total of four peptides. These peptides served as carriers for DNA, with the KH sequence being able to interact with plasmid DNA for gene delivery applications while also facilitating cellular uptake and endosomal lysis. To assess the feasibility and efficiency of peptide-mediated plasmid DNA (pDNA) delivery into human embryonic kidney (HEK) 293 cells, the authors custom-synthesized the four peptides and explored their DNA-binding affinities with pDNA constructs that encoded either Renilla luciferase (RLuc) or green fluorescent protein (GFP), all under the regulation of a mitochondrial-specific cyclooxygenase-2 (COX-2) promoter. Dynamic light scattering (DLS) and atomic force microscopy (AFM) were utilized for physicochemical characterization, revealing that most complex sizes fell within the 130–480 nm range, which was favorable for cellular uptake via the caveolae-mediated endocytic pathway. Transfection efficiency was quantified along with cell viability using a specialized RLuc assay. Intriguingly, Cytcox-KH/pDNA complexes at a low N/P ratio of 0.5 exhibited remarkably high transfection efficiencies without any negative impact on cellular viability. Using confocal laser scanning microscopy (CLSM) combined with MitoTracker Red and DAPI staining, the authors confirmed a strong intramitochondrial localization of GFP, distinguishing it from various cytoplasmic controls, including KH and Tat2 (a non-mitochondria specific cell-penetrating peptide) peptides complexed with pDNA. Circular dichroism (CD) spectroscopy was employed to establish the vital role of the α-helical structure in Cytcox-KH for targeted mitochondrial delivery, which further illustrated why the non-ordered OTC-KH was unsuccessful at transfection. As shown in this article, combining peptide blocks of different functionalities could prove to be a very intriguing and promising approach to better targeting modalities.

6. Clinical Translation and Precision Medicine

The precision medicine aims to reconcile the vast amount of genomic and functional data for accurate and targeted treatment of disease. As such, nanostructures and nanoparticles hold great promise for allowing further tunability and specificity and have already been investigated for multiple applications including cancer, cardiovascular disorders, and neurological conditions. Furthermore, they can enable real-time monitoring of disease progression and treatment response through imaging techniques, allowing for personalized and adaptive treatment regimens.

6.1. Stimuli-Responsive Systems

Stimuli-responsive biomaterials have been extensively researched as an emerging advanced class of molecules that can adopt different conformations from external triggers or stimuli. By incorporating desirable stimuli responses and unique self-assembly techniques, these biomaterials can be adapted towards many different specific applications.

For instance, peptides can be modified to be responsive to the pathological upregulation of matrix metalloproteinases (MMPs).[142] These systems offer targeted and efficient treatment modalities for diseases characterized by dysregulated MMP activity including tumorigenesis, inflammatory disorders, cardiovascular diseases, and neurodegenerative ailments. These peptides are specifically engineered to undergo cleavage in response to the heightened levels of MMPs in diseased tissues,

ensuring the localized and controlled release of encapsulated therapeutic agents. In cancer treatment, nanoparticles conjugated with peptides cleavable by MMP-2 and MMP-9 have demonstrated significant promise. The overexpression of these MMPs in the tumor microenvironment triggers the release of chemotherapeutic agents encapsulated within the nanoparticles directly at the tumor site. For example, PEGylated liposomes with MMP-2 sensitive peptides have been shown to enhance drug accumulation and efficacy in tumor cells.[143] The MMPs, specifically MMP-2, MMP-3, and MMP-9, play a crucial role in cardiovascular diseases as they not only signal the potential for MMP-responsive drug release during conditions like myocardial infarction but also present targets for direct therapeutic intervention.[142] Additionally, MMPs play a complex role in neurological disorders by both degrading amyloid-beta to prevent aggregation in diseases like Alzheimer's and increasing blood-brain barrier permeability, which can exacerbate neuroinflammation and neurodegeneration.[142] This innovative approach highlights the potential of peptide-based, stimuli-responsive drug delivery systems in addressing a wide range of complex medical conditions.

Stimuli-responsive peptides can also be seen in the area of supramolecular peptide gels, which exhibit remarkable adaptability and responsiveness to a range of environmental stimuli.[144] These gels, through their unique ability to respond to chemical, physical, and biological cues, have opened new avenues in various applications, especially in the biomedical domain. The pH changes critically influence peptide gelation. This sensitivity arises from changes in hydrogen bonding and the ionization states of amino acids. For example, a 20 amino acid peptide (ETATKAELLAKYEATHK) can adopt different conformations and self-assemble into various structures based on changes in pH, transitioning from α-helical to β-sheet or random coil structures, and forming aggregates or nanovesicles due to alterations in electrostatic and hydrogen bonding interactions influenced by protonation states of specific amino acids, such as lysine.[145] Redox stimuli are also important; peptides with cysteine residues can form or break disulfide bonds, undergoing structural transformations that affect the gel-sol state transition. For example, ferrocene's integration into peptides may significantly alter the gel's macroscopic properties depending on its oxidation state.[146] Physically, temperature and ultrasonication are key factors that may be exploited. Temperature-responsive peptides like Fmoc-D-Ala-D-Ala can form hydrogels when heated, facilitated by stronger intermolecular interactions.[147] Ultrasonication influences peptide particle aggregation, altering gelation by breaking intramolecular hydrogen bonds and forming new intermolecular interactions.

Other peptides are designed based on the temperature-sensitive transition from α-helix to β-sheet. Polypeptides and proteins such as elastin-like polypeptides, undergo a conformational change from random coiled to a β-spiral after being heated to a critical transition temperature.[148] Elastin-like polypeptides are more biodegradable than other polymer-based materials,[149] and are especially useful for injectable controlled-release,[150] thermally triggered targeting of tumors,[151] and protein purification tags.[152]

It is also possible for peptides and protein structures to be responsive to various enzymes. Biologically, enzymes act as specific triggers for gelation in certain peptide systems. Enzymes can transform precursor molecules into gelators, demonstrated by alkaline phosphatase catalyzing the dephosphorylation of Fmoc-tyrosine phosphate to form a hydrogel[153] This highlights the peptides' capacity to respond to precise biological signals.

6.2. Combination Therapy

A unique advantage of nanostructures compared to individual compounds is the possibility of combination therapy. Because there may be multiple underlying causes of a disease, precision medicine often necessitates an effective yet safe way to administer or address multiple therapeutic components at a time. Below we provide a few examples that have exploited this strategy in diverse disease settings.

Chimeric antigen receptor T cells, or CAR-T cells, are gaining interest for their ability to reprogram immune cells directly from a patient's body to present specific chimeric antigen receptors to target the desired antigens when ultimately reintroduced into the body.[156] Unfortunately, the process of isolating and reintroducing T cells into the patients is invasive and expensive. Rurik et al. tried to address this issue by formulating lipid nanoparticles containing CAR mRNA that target and transfect T cells directly in the patient body to treat cardiac injury, thereby exploiting aspects of gene delivery and immunomodulation at the same time.[157] Specifically, the lipid nanoparticles encapsulated mRNA for a CAR against fibroblast activation protein (FAP) and targeted CD5 through the conjugation of antibodies to mouse or human CD5, which is primarily expressed by T cells and a small population of B cells but is dispensable for T cell effector functions. Because cardiac fibroblasts are activated in cardiac injury and cause fibrosis, the authors hypothesized that reprogramming T cells to specifically kill activated fibroblasts could result in reduced fibrosis. The initial validation of the lipid nanoparticle's effectiveness to deliver mRNA was done through flow cytometry in murine T cells *in vitro*, and using mRNAs encoding for either GFP or FAPCAR in CD5-targeted nanoparticles the authors were able to show greater than 80% transfection rates, as compared to less than 10% transfection rate in the IgG isotype control-decorated nanoparticle. Surprisingly, the results translated well *in vivo:* the researchers first delivered luciferase mRNA intravenously in mice and found that splenic T cells were only bioluminescent in CD5/LNP-treated mice and not in the isotype IgG/LNP controls; both LNP treatments resulted in some luminescence in the liver which the authors attributed to hepatic clearance. Similar results were reported for CD5/LNPs loaded with Cre recombinase mRNA when injected into Cre-reporter mice where only successfully reprogrammed CD3+ cells (both CD4+ and CD8+ subsets) fluoresced green. They then validated whether the transfected T cells had therapeutic potential. First, *in vitro* testing showed that FAPCAR-T cells were able to trogocytose, or extract from the cell membrane and, therefore, damage cells, labeled FAP in

red fluorescent protein (RFP)-FAP overexpressed HEK293T cells. To test the validity of the FAPCAR system *in vivo*, they used a murine hypertensive model of cardiac injury and fibrosis. Like the *in vitro* results, FAPCAR-T cells with punctate FAP protein staining were observed in the white pulp region of the spleen in injured mice with CD5/LNP-FAPCAR injections, which was similar to injections of virally transduced T cells *ex vivo*. In addition to trogocytosis, FAPCAR-T cells improved cardiac function in injured mice as measured by normalized left ventricular end-diastolic and end-systolic functions back to uninjured levels, as well as by an improvement of the heart weight to body weight ratio, which is a measure of cardiac hypertrophy, all of which were supported by histologic analyses.

In a different example, a bioactive scaffold composed of two peptide amphiphiles with different biological signaling modalities was synthesized for the treatment of spinal cord injury[154] (Figure 4A). Specifically, laminin epitope IKVAV, which is known to promote differentiation of neural stem cells into neurons and extend axons, and fibroblast growth factor-2 (FGF-2) signal, via an FGF-2 mimetic peptide, which promotes cell proliferation and survival, were combined and allowed to form hydrogels at the spinal cord. The authors first optimized parameters for IKVAV signaling before incorporating the FGF-2 signal peptide amphiphile, and through multiple *in vitro* and *in vivo* experiments were able to demonstrate that both signals contribute to nerve damage recovery. In short, the IKVAV signal was able to successfully promote the differentiation of human neural progenitor cells by observing the induction of TUJ-1 as a marker for neuronal differentiation commitment; when combined with FGF-2 signals, more robust corticospinal axon regrowth due to increased angiogenesis and local neuronal cell survival was observed in a mouse model of spinal cord injury where the spinal cord undergoes contusive injury. Dual modality was achieved because both peptide amphiphiles were miscible and formed hydrogels: when injected at a molar ratio of 90:10 IKVAV peptide amphiphile with FGF-2 peptide amphiphile (PA) saline solutions, the hydrogel successfully gelled *in situ* at the damaged spinal cord and biodegraded gradually lasting up to 12 weeks. The optimization of the peptide sequences greatly depended on physical and modeling data on supramolecular motion determined by the different motion control peptide sequences of each of the two peptide amphiphiles separately and together, which we will not mention in great detail, but the authors pointed out that a more agile and plastic supramolecular scaffold could be more effective at signaling receptors in cell membranes undergoing rapid shape fluctuations.[154]

6.3. Bioimaging Applications

In addition to therapeutics, nanostructures can encapsulate compounds that facilitate tracking or imaging of various target tissues or organs. For example, RGD-conjugated human ferritin iron oxide nanoparticles have been formulated to allow *in vivo* MRI to evaluate vascular inflammation and angiogenesis in murine carotid arteries and abdominal aortic aneurysms.[158] As mentioned above, RGD exhibits a high affinity for the αvβ3 integrin, which is a suitable therapeutic and imaging target because it is elevated on neovessel endothelial cells and is found in high levels on atherosclerotic macrophages, suggesting important roles and potential in targeted imaging of vascular inflammation and angiogenesis. Previous work had illustrated the potential of human ferritin protein-cage nanoparticles for fluorescence imaging of vascular macrophages in murine carotid arteries. The RGD peptide was conjugated to these nanoparticles, enhancing their targeting capabilities for

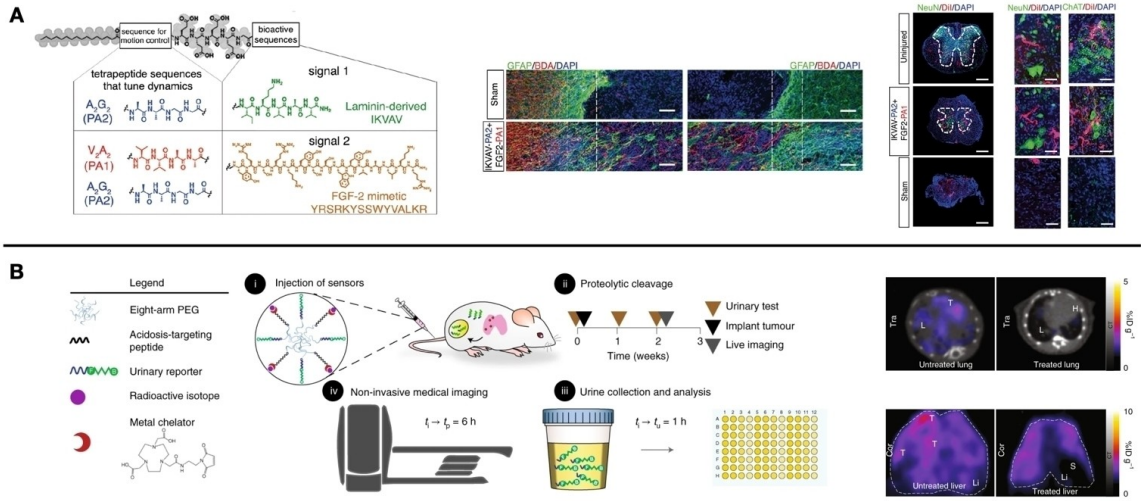

Figure 4. Examples of nanostructures for precision medicine. A) Two peptide amphiphiles with different bioactive signaling molecules were designed to form hydrogels for the treatment of neuronal loss. Fluorescent micrographs of spinal cord sections demonstrate that the platform promotes neuronal growth when compared to sham, where neurons are stained in green in both longitudinal and traverse spinal cord sections (partially adapted with permission from Álvarez et al.[154]). B) A polymer-peptide conjugate was developed for precision cancer diagnostics. Acidosis-targeting peptides conjugated to a metal chelator and radioactive isotope and urinary reporters conjugated to MMP-9 substrate peptides were multivalently conjugated to the polymer backbone for multimodal diagnostics approach with urine analysis and non-invasive PET-CT imaging (partially adapted with permission from Hao et al.[155]).

macrophages and angiogenic endothelial cells. MRI, possessing the benefit of clinical translatability and offering comprehensive vessel information, stands as a preferred imaging modality. Given that ferritin is an inherent human protein, it presents an advantage for clinical translation. To test the validity of the nanoparticles, two primary mouse models were employed, and in both models, nanoparticles were key: recombinant human heavy-chain ferritin (HFn), specifically modified to carry the RGD-4 C peptide, and its unmodified counterpart, were internally loaded with magnetite (Fe_3O_4) to serve as MRI agents. *In vivo* MRI scanning was performed post nanoparticle injection using a 3T MRI scanner. The image analysis focused on the percentage signal loss, either within the carotid lumen or the AAA's signal intensity. Preceding nanoparticle administration, MRI imaging showcased the diminished size of the ligated left carotid artery and a clear visualization of the aneurysmal site in the suprarenal aortic segment. After nanoparticle administration, significant T2 signal losses were observed in both RGD1 (targeted) and RGD– (non-targeted) groups, with the signal loss being more profound in the RGD1 group in both carotid and AAA models.

Similarly, in a different publication the fibrin-targeting CREKA (Cys-Arg-Glu-Lys-Ala) peptide was conjugated into supramolecular peptide amphiphile micelles containing gadolinium (Gd) for the use as molecular MRI contrast agents.[159] Two different peptide amphiphile structures, CREKA/DSPE-PEG2000-DTPA(Gd) and CREKA/DTPA-BSA(Gd) were used, where diethylenetriaminepentaacetic acid (DTPA) acted as a Gd chelator, and each were either conjugated to CREKA or left unmodified as non-targeting counterparts. The researchers showed both peptide amphiphile micelles bound to fibrin-containing clots *in vitro* and to plaques in atherosclerotic mice *in vivo* via MRI, suggesting their potential as contrast enhancers. In a follow-up paper, the authors used a similar CREKA/DSPE-PEG2000 platform to synthesize hybrid metal oxide peptide amphiphile micelles composed of either iron oxide or manganese oxide, each of which enhanced MRI contrast by reducing different relaxation times[160] and thus proposing a method to combine the benefits of both inorganic and organic nanoparticles. These hybrid particles exhibited biocompatibility, stability, and enhanced MRI contrast, but were further optimized to increase stability and permeability by adding components like phospholipids and cholesterol. Evaluations revealed that these additions enhanced nanoparticle interaction with cells and provided protection against protein binding and aggregation. Lastly, the CREKA moiety increased specificity to clots three to five times more than non-targeting counterparts, suggesting their use in the early preventive diagnosis of thrombi formation in atherosclerotic plaques.

Peptide nanostructures have also been used in the diagnosis and monitoring of cancer. Hao et al.[155] developed the Protease-Responsive Indicator for Sensing Metastasis (PRISM) platform, which leverages the inherent biochemical properties of tumorous microenvironments for non-invasive cancer detection and longitudinal drug-response monitoring (Figure 4B). PRISM employs synthetic biomarkers tailored to the unique hallmarks of cancerous tissues-specifically, extracellular acidification and matrix metalloproteinase 9 (MMP-9) activity. Through this, PRISM was able to provide a highly specific, dual-function diagnostic tool that could localize to tumors, including challenging tumor nodules in the lung and low-glucose-uptake malignancies. Specifically, the authors synthesized a modified version of pH low insertion peptide (pHLIP), which was shown to localize to acidic tumor microenvironments of both primary tumors and metastatic lesions due to tumor acidosis. This peptide was multivalently attached to an eight-arm PEG polymeric scaffold by click chemistry alongside MMP-9 protease substrates conjugated to fluorescently labeled reporters, which not only allowed for active tumor targeting but also allowed localization of the fluorescent signal. Interestingly, the fluorescently labeled reporters, when cleaved off from the protease substrates, could be measured in the urine of colorectal cancer mouse models for an additional non-invasive monitoring modality. One more modification was made to allow the PRISM platform to be useful in positron emission tomography-computed tomography (PET-CT) imaging: metal chelator NOTA was site-specifically conjugated to pHLIP to bind positron-emitting radionuclide ^{64}Cu. These ^{64}Cu-labelled PRISM nanostructures localized to lung and liver tumors and were able to overcome the limitations of conventional PET agent ^{18}F-fluorodeoxyglucose such as high background signal due to the increased uptake by metabolically active cells neighboring tumor tissues. Combining all the features, the PRISM platform allowed for localization to tumors, non-invasive measurement of reporters in urine, and non-invasive PET-CT imaging and tracking of tumors in real-time, which could also be combined with conventional drug treatment.

An additional delivery vehicle that can be used for cancer theranostics was reported by Meng et al.[161] They developed a superparamagnetic iron oxide nanoparticle (SPIO NP) system composed of a biomimetic H460 lung cancer cell membrane that has homotypic targeting ability to lung cancer and is decorated with therapeutic peptide TPP-1. TPP-1 binds to and inhibits PD–L1, resulting in checkpoint inhibition, which was conjugated to MMP-2 substrate peptides to allow for TPP-1 release. Biomimetic cell membrane-coated nanocarriers have emerged as game changers in the drug delivery domain owing to their inherent ligand recognition, prolonged blood circulation, and immune evasion properties. The researchers characterized cancer cell membrane vesicles derived from H460 lung cancer cells for potential homotypic targeting, which they then procured through hypotonic lysis, mechanical fragmentation, and differential centrifugation. Termed SPIO NP@M-P (SPIO NPs encapsulated in cancer cell Membrane and displaying TPP-1 and MMP-2 Peptides), these nanoparticles ensured targeted TPP-1 peptide release within tumors while significantly enhancing the half-life of the TPP-1 peptide, achieving a duration 60 times longer than the unbound peptide and thus promoting efficient reactivation of T cells and subsequent tumor growth inhibition. *In vitro* evaluations on H460 cells revealed minimal cytotoxicity, and *in vivo* mouse studies underscored the extended half-life and homologous targeting of SPIO NP@M-P. Additionally, SPIO NP@M-Ps allowed for aggregation at tumor sites and exhibited promising characteristics as a T2 contrast

agent for MRI, where higher Fe concentrations resulted in higher T2 relaxation rates.

6.4. Limitations and Potential Solutions

One limitation to the advancement of peptide nanostructure research is in fact its high modularity. Some structures may follow specific scaling properties,[54] but with the variety of peptide sequences and formulations reported in the literature, it is hard to agree on universal properties for all such nanostructures. In this regard, much can be learned from the field of chemogenomics and small molecule drugs. Chemogenomic screens are primarily focused on identifying the phenotypic effects of small molecule leads on target gene families and can be carried out in a high throughput manner. In doing so, chemogenomic screens yield 'targeted therapeutics' for their modulatory effects on specific molecular targets.[162] Still, there are limitations on the information we can get from chemogenomics: first, small molecules usually target 'druggable genes' that encode proteins such as G-protein coupled receptors and kinases;[162,163] second, these screens are usually performed in *in vitro* bioassays that might not account for the natural cellular heterogeneity of the target organ or tissue and do not by themselves target a specific site; lastly, chemogenomic results come mostly from phenotypic data, and thus must be validated with complementary techniques such as RNAi or CRISPR-Cas9 approaches.[163] Considering these limitations, peptide nanostructures could offer synergistic solutions: nanostructures could be used to specifically deliver small molecules or gene editing components to a target organ or tissue to validate and/or enhance results from such screens to specific molecular targets. Additionally, we have provided multiple examples of the breadth of therapeutics that could be encapsulated in peptide nanostructures: genomic information from chemogenomic screens could be harnessed without limiting the cargo to small molecule drugs while still taking advantage of the additional targeting strategies described in this review.

As discussed above, the number of drug candidates is constantly increasing, but only a few are eventually approved for clinical use. High throughput screens have also identified multiple drugs that exhibit therapeutic effects on non-intended disease targets. Understandably, small molecules can inherently exhibit off-target effects, even in previously uncharacterized targets.[163] Although these off-target effects have been historically considered as detrimental, current advances in pharmacology and drug delivery platforms could allow for the specific and selective delivery of a single drug to different organs or tissues for different purposes, which is now broadly termed as drug repurposing.[164].[164] Because peptide nanostructures can be highly modular, drugs that have already undergone clinical trials or even been approved could be repurposed, thereby possibly cutting the need for having to develop new drugs for a particular function or need. Additionally, advancements in microfluidics now allow for the automation and miniaturization of high-throughput screens, facilitating clinical prediction and testing of therapeutics.[165]

Closely related to the expansive amount of data produced from such screens is the challenge of comprehensively analyzing and managing such data. Fortunately, advances in computing and artificial intelligence are shedding insights on how to integrate datasets and rationally interpret them.[166] Molecular dynamics simulations and other computational modeling and machine learning approaches, while still costly, have been already reported specifically in the field of nanomaterials design for drug discovery and precision medicine.[10] Likewise, machine learning may play pivotal roles at each stage of the drug development process, including peptide and protein drugs, from the diagnosis of disease,[167] prediction of possible drug candidates in disease-specific contexts to prediction of suitable delivery methods, drug physicochemical properties and pharmacological outcomes,[12,168] which may be integral to the faster development of successful candidates while lowering the overall costs involved in the process.

Most importantly, machine learning and artificial intelligence can also be used to identify novel peptide sequences that can be designed for specific functions. For instance, deep learning was recently used to identify a cell-penetrating peptide sequence that broadly enhanced the uptake of conjugated molecules from a chimeric library of more than 600 morpholino-CPP conjugates.[169] Primarily, machine learning was applied to identify sequences that contained less arginine sequences as high arginine content usually correlates with increased toxicity. In a different example, machine learning was used to identify hexapeptide AMPs from the entire search space of 64 million sequences; even after pre-filtering through empirical selection for small amphipathic peptides that are net positive at physiological pH, the resulting candidate pool consisted of 3.93 million sequences.[170] After multiple rounds of filtering, the top 10 ranking peptides were validated *in vitro*, and after further validation, one candidate was tested *in vivo* and showed similar effects to penicillin in a mouse acute bacterial pneumonia model. The strength of machine learning is even more notable when searching for longer peptide sequences; for nonapeptides, pre-filtering through empirical selection resulted in 512 billion sequences. The researchers reported that the best nonapeptide sequence could be identified in less than a month, where the initial pre-filtering and wet laboratory validation alone occupy most of that duration.

7. Summary and Outlook

In this review, we presented an overview of recent efforts to deliver peptide and non-peptide therapeutics via nanostructures. We introduced the different chemistries used to form such structures, methods in which these can be modified or decorated depending on the desired application, and examples on specific disease models both *in vitro* and *in vivo*. The scope of what can be termed as precision medicine is continuously growing as new chemical and genomic targets are being developed. Thus, despite an increase in the number of potential

therapeutics to be delivered, additional screening controls and parameters are important as these technologies move to the market and the clinic. An additional roadblock to the development of new nano-delivery platforms may be the fact that *in vitro* and *in vivo* results do not always correlate well,[159,171] where a nanostructure that would work *in vivo* does not *in vitro* or vice versa: provided that the end goal of all therapeutics is translation into the clinic, this would be yet another hurdle to overcome. For this purpose, lipid, polymer or peptide components that confer nanoparticles organ tropism could be further explored to facilitate *in vivo* translation. For example, SORT (Selective ORgan Targeting) lipids supplemented to conventional lipid nanoparticles were recently reported to modify organ tropism.[171,172] Peptide and protein therapeutics could be assembled with such lipids, or investigations on whether different peptide sequences could also modify tissue tropism could further aid in enhanced therapeutic efficacy.

We also touch on the idea that data from high-throughput screening and chemogenomics may inform our course of development of new peptide nanostructures, precisely as therapeutics are often delivered in their native form without a delivery vehicle. The pharmacokinetic profiles of drugs may be obtained more quickly with the development of artificial intelligence and high throughput screening and diagnostics, platforms which would be beneficial to adopt also in the screening of higher order nanostructures both on the benchtop and in model organisms. On the other hand, peptides can also be implemented in the development of novel therapeutics: advancements in next-generation sequencing and artificial intelligence could elucidate how peptides can be further used to deliver new therapeutics or target precise tissues and organs, possibly even in personalized medicine approaches. In summary, we have introduced innovative and promising technologies that highlight basic principles that could be applied in the development of multiple delivery modalities.

Conflict of Interests

The authors declare no conflict of interest.

Keywords: Peptides · Drug Delivery · Nanostructures · Targeted Delivery · Precision Medicine

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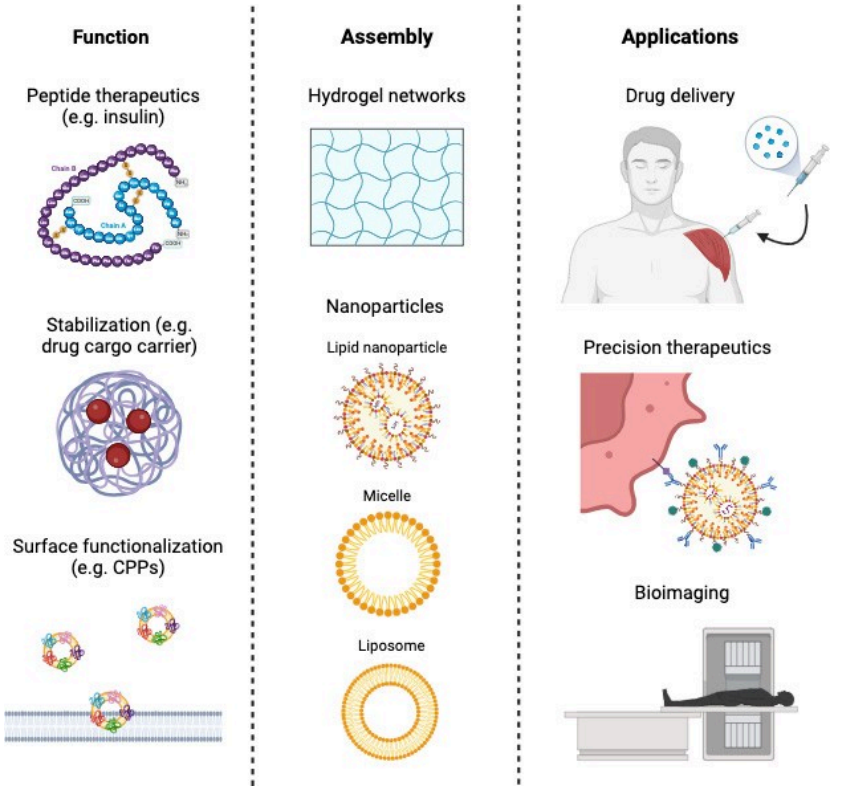
Manuscript received: December 8, 2023
Revised manuscript received: February 5, 2024
Accepted manuscript online: February 26, 2024
Version of record online: ■■, ■■



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Therapeutic Peptides, Proteins and their Nanostructures for Drug Delivery and Precision Medicine

Peptide nanostructures with tunable structural features, multifunctionality, biocompatibility and biomolecular recognition capacity enable development of targeted drug delivery tools for precision medicine applications. In this review article, we present various techniques employed for the synthesis and self-assembly of peptides into nanostructures, design strategies utilized to enhance their stability, drug-loading capacity, and controlled release, and applications in precision medicine.