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DESIGN, SYNTHESIS, AND IN VITRO EVALUATION OF PEPTIDE-BASED DRUG DELIVERY SYSTEMS

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ABSTRACT

Peptide-based drug delivery systems (DDS) have garnered significant attention due to their ability to enhance the targeting, stability, and controlled release of therapeutic agents. This study focuses on the design, synthesis, and in vitro evaluation of peptide-based DDS, aiming to improve the efficacy of drug therapies while minimizing systemic side effects. Peptides are chosen for their biocompatibility, selectivity, and ability to bind specifically to cellular receptors, making them ideal candidates for targeted drug delivery. In this work, a series of peptide conjugates were synthesized by coupling therapeutic drugs with targeting peptides, ensuring optimal drug release profiles.

The synthesis involved solid-phase peptide synthesis (SPPS) and the conjugation of peptides to drug molecules, followed by characterization using techniques like high-performance liquid chromatography (HPLC), mass spectrometry (MS), and nuclear magnetic resonance (NMR) spectroscopy. In vitro evaluation of the drug delivery systems was conducted to assess their release kinetics, cytotoxicity, and cellular uptake. The drug release profiles were monitored under different physiological conditions to mimic real-life scenarios, ensuring controlled release over extended periods.

The results demonstrated that the peptide-based DDS exhibited enhanced drug solubility, stability, and selective cellular uptake, with controlled release profiles over time. Additionally, the conjugates showed minimal cytotoxicity, indicating their potential for safe therapeutic applications. The findings from this study suggest that peptide-based drug delivery systems hold great promise in revolutionizing the treatment of various diseases by providing targeted therapy, improving patient outcomes, and minimizing adverse effects. Further studies, including in vivo testing, are needed to validate these results and explore clinical applications.

I. INTRODUCTION

A large number of compounds fail to progress through the various stages of preclinical and clinical studies due to a number of reasons, including but not limited to, high cytotoxicity, poor pharmacokinetic rate and inefficient site-specific targeting. Pharmaceutically active substances at physiological conditions should be able to overcome biological obstacles such as albumin binding and aggregation, insolubility, biodegradation/metabolism, the low

permeability via vascular endothelial cell layers, rapid excretion by the kidney, inefficient cellular internalization and undesirable immunogenicity [1]. These issues create a narrow therapeutic window and as a consequence, lead to dismal in vivo performance. The development of a novel drug, including all stages of clinical studies, costs about USD 2.2 billion [2]. The use of novel drug delivery systems (DDSs) that improve the properties of a cell membrane's penetration may provide an opportunity of recycling drug candidates, which have previously demonstrated the above-mentioned disadvantages. Moreover, repeated drug administration raises the cost and, in many cases, causes undesirable side effects. DDSs based on synthetic stimuli responsive copolymers are widely discussed in recent reviews, including thermoresponsive [3,4], pH responsive [5–7] and modified natural polymers [8] that improve the stability, the effectiveness of pharmacokinetics and the tolerability of existing substances, concurrently mitigating their off-target toxicity.

A comprehensive review of nanocarriers based on various types of lysosomes, solid lipid nanoparticles, dendrimers, polymeric micelles, virus-based nanoparticles, inorganic and organic/inorganic hybrid nanocarriers discusses advances in the application of carriers with non-immunogenic and biodegradable architecture for having optimal pharmacokinetic and pharmacodynamic parameters of the drugs [9]. Some liposome-based DDSs have been approved by the U.S. Food and Drug Administration (FDA), such as liposomal doxorubicin (Doxil®) and liposomal amphotericin B (Ambisome®) [10]. A recent review covers standardization parameters of therapeutic peptides and compares the monographs of different Pharmacopeias all over the world, which is an important and obligatory stage for entering the market. Thus, to date, there are about 60 peptides that have reached the market, more than 150 peptides are in active clinical trials and about 260 peptides are currently being tested in humans and over 400 peptides in nonclinical studies [11]. There is also a classification of DDSs based on pharmaceutically active substance delivery to a specific organ, for example, growth factor or cytokine delivery to accelerate and improve tissue repair and regeneration [12–16]. Numerous research studies indicate that biodegradable natural, semi natural, synthetic and hybrid polymers serve as a milestone in the design and development of innovative DDS paradigm, improving the management and healing of damaged tissue, decreasing side effects and improving the pharmacodynamics of the substance [17,18].

On some occasions, extracellular vesicles derived from cells can be utilized for effective delivery of various substances such as proteins, lipids and genetic materials (messenger RNA (mRNA), microRNA, other small non-coding RNA and genomic DNA) to the target cell or organ [19]. The drawback of most of the above-mentioned systems is their poor control of delivery to some target organs or cells, slow pharmacokinetic or release rate, harmful degradation products and unsatisfactory penetration via the blood-brain barrier. There are a number of papers related to peptide-based carriers, which were not discussed in previous review papers, thus filling this gap will generate new ideas for the creation of efficient DDSs and provide such qualities as utilization of a prodrug where a small

peptide sequence is covalently conjugated to the active substance disguising its pharmacological activity until it is triggered by a disease-specific agent at the desired site [10]. Encapsulating the drug in a peptide-based DDS dictates the pharmacokinetics and pharmacodynamics through its unique physico-chemical properties and the possibility of the use of an implantable drug-eluting depot. Cooper and colleagues have analyzed peptide-based carriers and the modification of protein sequence to slowing down renal clearance of carriers loaded with drugs. Moreover, they performed a comprehensive analysis of data related to antibody–drug conjugates, which is also a promising trend in targeted therapy [20]. This review highlights the design of drug delivery carriers based on peptides and their advantages compared to synthetic polymer-based DDSs, hybrid particles and composite carriers due to less of a response from the immune system, as well as the absence of toxic products of degradation and the possibilities of modulating the entrance of the carrier via the cell membrane and of designing a specific high affinity sequence that provides efficient binding with the target. Furthermore, the functional efficiency of the peptides and their contribution to the overall therapeutic efficiency of DDSs are reviewed and discussed.

II. LITERATURE SURVEY

Peptide-based drug delivery systems (DDS) have gained significant attention in recent years due to their potential to address several limitations of conventional drug delivery systems. The ability of peptides to target specific receptors or tissues, combined with their biocompatibility, stability, and biodegradability, makes them ideal candidates for the design of advanced DDS. This section reviews the current state of peptide-based DDS, focusing on their design, synthesis, and evaluation, as well as their applications in drug delivery.

1. Peptide-Based Drug Delivery Systems: Concept and Design

Peptide-based DDS are primarily designed to overcome challenges such as poor drug solubility, rapid clearance from the body, and non-specific distribution. Peptides, often derived from natural or synthetic sources, can be used to functionalize drug molecules, enhancing their solubility and pharmacokinetic properties. Peptides are chosen based on their affinity for specific cellular receptors, which allows for selective targeting of disease sites, including tumors, inflamed tissues, and infected areas.

A key aspect of peptide-based DDS is the conjugation of therapeutic agents to targeting peptides through stable chemical linkages, ensuring effective drug delivery. The design of these conjugates requires consideration of the peptide’s size, charge, stability, and ability to cross cellular membranes. Several strategies have been employed to improve the performance of peptide-based DDS, including the use of pegylation, lipidation, and the incorporation of nanoparticles or liposomes to enhance stability and reduce clearance rates.

2. Synthesis of Peptide-Based DDS

Peptide synthesis methods have evolved significantly over the years. Solid-phase peptide synthesis (SPPS) is the most widely used technique for the production of peptides. This method allows for the efficient, high-yield synthesis of peptides

with precise amino acid sequences. In DDS development, peptides are typically conjugated to drugs or other delivery agents through covalent bonds, which are stable enough to ensure drug retention during circulation but can be cleaved under specific conditions (such as low pH or enzymatic activity) to release the drug at the target site.

A variety of methods have been developed for the synthesis of peptide-drug conjugates, including click chemistry, carbodiimide coupling, and enzyme-mediated conjugation. These approaches ensure the stability of the peptide conjugates while allowing for the controlled release of the drug.

3. In Vitro Evaluation of Peptide-Based DDS

The in vitro evaluation of peptide-based DDS involves assessing key parameters such as drug release kinetics, cytotoxicity, cellular uptake, and receptor binding affinity. Drug release studies are critical to understanding the stability and controlled release profiles of peptide-drug conjugates. Release kinetics are often evaluated under physiological conditions to determine the efficiency of the system in delivering the drug over time.

Cytotoxicity testing is conducted to evaluate the biocompatibility of the peptide conjugates. This includes assessing the effects of the conjugates on cultured cell lines to ensure that they do not cause excessive toxicity to healthy cells. Cellular uptake studies are also essential to assess the ability of the peptide conjugates to penetrate cell membranes and deliver drugs to the target cells. Common techniques for these studies include fluorescence microscopy, flow cytometry, and confocal imaging.

Furthermore, receptor binding assays are conducted to ensure that the peptide conjugates can specifically target cells expressing the corresponding receptors. These studies are important for demonstrating the specificity and effectiveness of the peptide-based DDS in targeting disease sites.

4. Applications of Peptide-Based DDS

Peptide-based DDS have shown promise in several therapeutic areas, including cancer therapy, gene delivery, and the treatment of autoimmune diseases. In cancer therapy, peptides are often used to deliver chemotherapeutic drugs or small molecules directly to tumor cells, reducing systemic toxicity and enhancing therapeutic efficacy. For example, peptides that specifically bind to overexpressed receptors on tumor cells can be conjugated to anticancer drugs, ensuring that the drugs are delivered selectively to the tumor site.

In gene therapy, peptides are used to deliver nucleic acids, such as DNA or RNA, into cells. This has the potential to treat genetic disorders by introducing functional genes into cells, either to replace defective genes or to silence harmful ones. The use of peptides in gene delivery offers several advantages, including the ability to overcome barriers such as the cellular membrane and endosomal escape.

In the treatment of autoimmune diseases, peptides can be used to deliver anti-inflammatory drugs or immunosuppressive agents directly to the site of inflammation, reducing the need for systemic treatment and minimizing side effects.

5. Challenges and Future Directions

Despite their promising potential, peptide-based DDS face several challenges. One of the main issues is the stability of peptides in vivo, as they are susceptible to enzymatic degradation by proteases. Strategies such as peptide modification, encapsulation in nanoparticles, or the use of cyclic peptides have been developed to address this issue. Additionally, the cost of synthesizing peptides and peptide-drug conjugates remains a significant barrier to large-scale production.

Future research will likely focus on improving the stability, targeting efficiency, and scalability of peptide-based DDS. New methods for conjugation and drug loading, as well as advances in nanotechnology, may further enhance the effectiveness of these systems. Additionally, the integration of peptide-based DDS with other drug delivery platforms, such as lipid nanoparticles or micelles, could further improve their performance in clinical settings.

6. Conclusion

Peptide-based drug delivery systems represent a promising strategy for improving drug efficacy and targeting in the treatment of various diseases. The ability to design and synthesize peptides that specifically target diseased tissues, combined with their biocompatibility and stability, makes them an attractive option for advanced drug delivery. Although there are challenges to be addressed, such as peptide stability and large-scale production, ongoing research in peptide synthesis, characterization, and drug delivery will continue to drive innovation in this field. The successful development of peptide-based DDS has the potential to revolutionize personalized medicine and improve therapeutic outcomes for patients with complex diseases.

III. Cell Penetrating Peptides

3.1. Molecular Mechanisms of Delivery of Cell Penetrating Peptides

Certain peptides are regarded as safe and effective DDSs because they may penetrate cells without compromising the integrity of the cellular membrane. Cell penetrating peptides (CPPs) are the typical classification for this family of peptides [21]. CPPs encompass residues 48 to 60 and are first obtained from the α-helical domain of the TAT protein, which is encoded by the HIV1 virus [22]. CPPs are generally cationic, short peptides (less than 30 amino acids) with the ability to conjugate medicinal compounds. These days, over 1800 distinct sequences with experimental validation may be found in CPPs [23]. Endocytosis and direct translocation are the two primary cellular uptake routes that allow CPPs to enter cells [22]. Nonetheless, there are two routes that some CPPs might enter the cell (Figure 1). A bovine lactoferricin L6 CPP, for instance, can be internalised via endocytosis, according to newly published research, but it can also be internalised by direct membrane translocation if polyhistidine peptides are added to the complex [24,25].

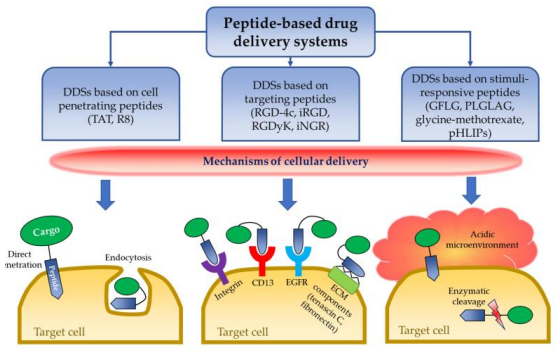

Figure 1. Peptide-based drug delivery systems.

Three distinct pathways—clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), and macropinocytosis—are involved in the energy-dependent process of endocytic internalisation of CPPs [26]. Numerous growth hormones, such as platelet-derived growth factor, macrophage colony-stimulating factor-1, and epidermal growth factor (EGFR), among others, can stimulate macropinocytosis, a typical nonspecific mode of cellular absorption [27]. Actinic cytoskeletal components first reorganise the cytoskeleton, which causes the cellular membrane to invaginate. A sheet-like pocket and giant endocytic vesicles that capture extracellular fluid and particles in macropinosomes are then formed [28]. Nakase and associates showed that the start of macropinocytosis is similarly mediated by a single transmembrane domain protein known as syndecan. Actin-rich peptides may be taken up by macropinocytes as a result of syndecan multimerization and actin polymerisation that follow peptide contact with the cellular membrane [29]. The primary mechanism for polyarginine, TAT, and NickFect51 absorption is macropinocytosis [30, 31]. Through actinic cytoskeletal elements, CPPs carrying cargo can attach to the cellular membrane in CvME and start the clustering of caveolin-1 proteins [28]. This results in the internalisation of the vesicle by the development of flask-shaped invaginations and further phosphorylation of caveolin-1 [26]. TAT, proline-rich CPPs, the PepFect14/DNA conjugate, and the p18 and p28 azurin fragments are all absorbed by this route [32]. According to a recent research, β-glycosidase, poly (I:C), and plasmid were successfully delivered into HCT116 cells via CvME by a new chicken anaemia virus-derived CPP known as CVP1 [33]. Lastly, CME starts when peptides bind with certain cell surface receptors to produce vesicles in phosphatidylinositol 4,5-biphosphate-rich areas of the plasma membrane, which initiates endocytic processes. Clathrin assembly to the plasma membrane is then started when an adapter protein attaches to phosphatidylinositol 4,5-biphosphate [34]. The clathrin-covered membrane then invaginates towards the cytoplasm, resulting in the creation of a coated pit. GTPase known as dynamin cleaves the membrane neck that forms as the pit continues to change into a spherical bud. Clathrin-coated vesicles that are released farther quickly lose their clathrin protein covering and are sent to early endosomes [35, 36]. After being transported to the nucleus by microtubules, an early endosome develops into a late endosome. Then, late endosomes transport their cargo to low-pH lysosomes and organelles [28, 32]. TAT peptide, oligo-arginine, and anionic CPPs have all been shown to include CME in peptide transport to the cell [32,37].

Peptide translocation is an energy-independent activity that takes place at low temperatures and without receptor involvement. Positively charged CPPs first interact with the phospholipid bilayer and negatively charged membrane components [38]. Four distinct pathways—the barrel stave model, toroidal pore model, carpetlike model, and inverted micelle model—were put up for further CPP internalisation by direct translocation [39]. The hydrophilic portions of CPP align with the lipid bilayer surface of the cell membrane in the barrel stave model.

Furthermore, the outer cellular membrane's surface alters to create perpendicular holes with hydrophilic CPP residues covering the pores' inside environment when a high pH and an adequate quantity of peptides are present [38, 39]. The creation of pores after peptide interaction with the polar head groups of lipids within the cellular membrane is a characteristic of the toroidal pore model. Both the injected peptides and the hydrophilic phospholipid cell membrane produce the hydrophilic walls of the toroidal pore [39]. In the carpet-like model, the cell membrane is sufficiently covered by CPPs to allow hydrophilic peptide groups to interact with the membrane's phospholipid head groups without internalising into the hydrophobic core. The cell membrane reorganises as a result of subsequent pep-tide rotation, creating a temporary hole in the membrane [39]. Lastly, the creation of micelles between the outer and inner membrane bilayers upon CPP contact with the cell membrane is what defines the inverted micelle concept. Because of their hydrophobic surface, hexagonal micelles envelop CPPs and facilitate the passage of hydrophilic substances conjugated to the peptide. Additionally, upon contact with the inner member bilayer and micelle destabilisation, peptides containing cargo are released into the cytosol [32].

3.2. Drug Delivery Systems with CPPs

The inability of medications to enter cells is a significant barrier to drug delivery. However, the discovery of cell-penetrating peptides like HIV1-TAT has allowed for the removal of this restriction. Additionally, novel DDS have been developed that effectively utilise TAT's special cell-penetrating ability. Since leukemic cells in lymphoblastic leukaemia primarily depend on an external supply of asparagine, Kwon and colleagues developed a TAT-asparaginase complex that may be useful against this kind of leukaemia [40]. While the results of earlier studies showed that TAT domains demonstrated comparable ability to translocate a variety of drugs across the membranes of many cell types, their experiments showed that the TAT-asparaginase complex was able to penetrate both the hepatocyte cell line (HeLa) and the MOLT-4 tumour cell line with remarkable efficiency. TAT-protein complexes are extremely effective therapeutic agents due to their penetrating capabilities, but this also makes them more harmful to healthy cells and can result in undesirable side effects. The scientists combined the ATTEMPTS DDS with the TAT-asparaginase combination in order to lessen the potential harm to healthy tissues. Due to the electrostatic interaction between negatively charged heparin and oppositely charged surface functional TAT groups, which totally prevents TAT from entering, the complex was first rendered inactive [40]. When the goal is accomplished, the bloodstream is filled with positively charged protamine, which binds to heparin more strongly than TAT [40,41]. As a result, protamine attaches itself to heparin, dislodging TAT-asparaginase and restoring TAT's penetration capacity, which allows asparaginase to enter cells. To investigate if the heparin-protamine regulation permits the regulated release of the TAT-asparaginase, HeLa and MOLT-4 cell lines were employed. A particular antibody that targets the complex to the tumour cells is also included in the original ATTEMPTS model. Kwon and colleagues, however, performed in vivo tests by directly

injecting asparaginase-encapsulated L5178Y cells into the mice, simulating cellular absorption of asparaginase instead of employing the original antibody-directed approach. The asparaginase-encapsulated tumour injected group's survival time was 1.7 days longer than the tumour injected mice's overall survival time [40]. Furthermore, Shin et al. have developed and evaluated the PTD-modified ATTEMPTS model in vivo using carcinoembryonic antigen monoclonal antibodies to target TAT-Gel (gelonin fusion chimaera) to LS174T cells exhibiting high expression of the carcinoembryonic antigen [42]. Protamine injection into the TAT-Gel-heparin-pretreated mice resulted in a 65% inhibition of tumour development, according to the results.

An oral charge reversible DDS for piercing mucous and epithelial barriers was created by Wu and associates [43]. Using polyethylene glycol (PEG) bonds, the system depicts poly(lactic-co-glycolic acid) (PLGA) nanoparticles coupled with octa-arginine peptides (R8) and phosphoserines (Pho) (P-R8-Pho NPs). The system's slightly net negative charge (zeta potential −2.4 mV) prevents electrostatic interactions with negatively-charged mucin, allowing for mucus penetration [44]. Conjugated cationic R8 and anionic phosphoserine moieties mutually cancelled their charges, resulting in the system's low net negative charge. However, the charge reversal occurs because the system needs positively charged functional groups in order to interact with and pass through the negative plasma membrane of the epithelial cells. Intestinal alkaline phosphatase, which is produced in the intestinal epithelium, cleaves the phosphate moiety on phosphoserine, causing the charge reversal [45]. Because arginine-rich R8 peptides function as cell penetrating agents and enable the system to cross the epithelial barrier, the system's total charge increases to a positive (+7.4 mV). As a result, the system's charge change, which is around 9.8 mV, is caused by the intestine alkaline phosphatase environment. The system has an excellent mucus penetrating ability of 14.8 × 10−6 cm/s and 12.6 × 10−6 cm/s, respectively, which is nearly as good as that of traditional PEGylated nanoparticles. In vivo research using diabetic mice employed insulin as a model medication [43]. Through the release of glucagon, transcriptional control of key gluconeogenic genes like PCK1 and G6PC, and activation of signalling pathways involved in gluconeogenesis, such as the PI3K and MAPK pathways, insulin, a hormone secreted by pancreatic beta cells, controls the synthesis of glucose [46, 47]. While oral delivery of free insulin did not lower blood glucose levels when the same 50 IU/kg dosage was applied in each case, oral administration of insulin-loaded P-R8-Pho NPs showed a 32% reduction in blood glucose levels after 3 hours (Table 1). Additionally, the system's relative bioavailability was 6.0 ± 0.9%, but free insulin's was just 0.40 ± 0.15% [43].

There have also been reports of R8-conjugated DDSs penetrating cancer cells. Li and colleagues engineered the delivery of the CRISPR/Cas9 complex for the treatment of pancreatic cancer. R8-conjugated cationic liposomes were used to encapsulate the compound [48]. The CRISPR-Cas9 combination includes the guide molecule sgRNA and the DNA endonuclease Cas9, which cleaves the target double-stranded DNA. Because it facilitates an epigenomic repair of mutant

cancer genes, CRISPR-Cas9 is being employed in a number of anti-tumor applications, including oncolytic virotherapy, cancer immunotherapy, and stromal-targeting treatments [49]. Furthermore, the delivery of short interference RNAs to treat liver cancer was investigated using R8 peptide-linked DDSs. By cleaving particular sections of targeted messenger RNAs, a process known as RNA interference, small interference RNAs promote cancer-specific gene silence [50,51]. In HepG2 tumor-bearing mice, he and his colleagues observed a tumour volume inhibition of around 80% [52]. R8 conjugated DDSs were also used in the treatment of wounds. According to Li and colleagues, R8-mediated collagen/chitosan gel administration promoted cutaneous wound healing by promoting angiogenesis, granulation tissue development, and collagen deposition. [53].

Table 1. R8 peptide conjugated DDSs.

Delivery Vehicle	Therapeutic Agent	Mechanism of Action of the Therapeutic Agent	Application	Outcome	References
PLGA nanoparticles	Insulin	Secretion of glucagon, transcriptional regulation of gluconeogenic genes	Diabetes mellitus treatment	Blood glucose reduction by 32% in three h	[43]
Cationic liposomes	CRISPR/Cas9	Correction of mutated tumor genes	Pancreatic cancer treatment	~70% tumor volume suppression	[48]
Upconversion nano-onions	Small interference RNA	Gene silencing through the cleavage of messenger RNAs	Liver cancer treatment	~80% tumor volume inhibition	[52]
Collagen/chitosan gel	Collagen/chitosan	Increased angiogenesis, collagen deposition, granulation tissue formation	Wound healing	98 ± 4.7% surface healing after 2 weeks	[53]

IV. TARGETED DELIVERY OF PEPTIDES

4.1. Molecular Mechanism of Targeted Delivery of Peptides

Paul Ehrlich initially proposed the concept of targeted medication administration at the start of the 20th century [54]. The idea originated from the fact that diseased tissue contains a variety of intricate cellular and non-cellular components [55], which may be targeted by medications that function as "magic bullets," removing diseased cells only while sparing healthy ones, thus increasing drug use and lowering adverse effects [56,57]. Since then, the idea of targeting has been revised and separated into active and passive targeting, and hundreds of distinct DDSs with side-directed accumulation capabilities have been described. Drugs accumulate in sick locations in passive targeting because of the intrinsic features of DDSs, such as their size, shape, and charge, as well as the unique characteristics of the targeted areas, such as lymphatic drainage and local vasculature. For instance, lymphatic drainage is either missing or compromised at tumour locations, while the surrounding vasculature is leaking [58]. Under such circumstances, DDSs show the so-called enhanced penetration and retention effect, which permits the preferential accumulation of large molecular weight polymers and nanoscale particles with a diameter of around 20–500 nm within the tumour tissue [59]. By affixing receptor-specific ligands to the medication or drug carrier, active targeting is subsequently receptor-directed. Peptides are often utilised targeting agents in medication delivery because they are natural ligands for a large number of receptors in human bodies. Because tumor-specific markers are overexpressed, targeting peptides are most frequently used as a delivery mechanism to target different tumour cells or tissues [60]. This class of peptides has a number of potential molecular targets, including as integrin receptors, aminopeptidase N, extracellular matrix (ECM) constituents, and EGFRs (Figure 1).

By controlling several stages of tumour cell motility and invasion, integrins regulate the development and survival of tumour cells throughout their escape and infiltration into blood or lymphatic vessels

[61]. The 24 heterodimeric cell-adhesion receptors that make up the integrin receptor family each have a mix of α and β subunits [62]. One of four integrin classes known as RGD-binding receptors is capable of binding to the Arg-Gly-Asp (RGD) peptide. Of the eight members of RGD receptors (αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, α8β1, α5β1, and αIIbβ3), αvβ3, αvβ5, α5β1, and αvβ6 are known to have a role in the development of malignancies and their subsequent metastases. Additionally, tumour cells' blood vessels overexpress the αvβ3 heterodimer [63]. RGD peptides have been demonstrated to bind to integrin receptors and suppress the production of ECM glycoproteins including fibrinogen and vitronectin, which reduces cell adhesion and promotes the growth of tumours [64]. Additionally, integrins experience CvME and CME upon ligand contact. This implies that drug conjugates that target peptides can deeper enter tumour cells, reducing their toxicity to healthy cells [65].

4.2. DDSs with Targeting Peptides

The RGD tripeptide is a well-known targeting peptide sequence (Figure 1). The tripeptide binds a variety of integrin receptors, including αvβ3 and αvβ5, which have been shown to express themselves more often in the endothelium of neonatal solid tumour arteries [79]. Up till now, a wide range of RGD peptide modifications have been discovered, including but not restricted to well-known examples like cyclic iRGD, cRGDyK, cRGDfC, cRGDfK, and cN-Me-VRGDf [80]. To increase the peptide's stability against proteolytic degradation and its affinity for integrin receptors, the sequence is lengthened with the right amino acids and cyclisation is introduced [81]. Two disulphide bridges between C2-C10 and C4-C8 cyclize RGD-4C (ACDCRGDCFCG), which has a 200-fold greater affinity for αvβ3 or αvβ5 than linear peptides, according to Zhou and colleagues [82]. However, a wealth of studies using unaltered RGD sequences also demonstrated the tripeptide's targeting capabilities and comparable positive outcomes. RGD-conjugated DDS is demonstrated by Cao and colleagues' research on exosome delivery of vanadium carbide quantum dots. Fluorescence spectroscopy was used to compare the RGD-conjugated systems with non-conjugated exosomes. The results showed that RGD enhanced targeting since it resulted in a larger accumulation of DDSs in tumour locations and a lower accumulation in the liver, kidney, spleen, and heart [83]. After creating an RGD integrated red blood cell-based multimodal probe (RBCp) for photodynamic therapy and fluorescence imaging-guided tumour surgery, Wang and colleagues were able to increase the photoacoustic signal at the tumour sites in mice treated with the RGD-conjugated system by 2.1 times compared to mice treated with the system without RGD. RBCps with RGD were said to exhibit a tumor-to-liver ratio of the system accumulation that was almost twice as high as that of RBCps without RGD [84].

It is possible to see overexpression of integrins specific to the RGD motif outside of tumour locations. Tian and associates found that the vasculature in ischaemic areas had a significant uptick in avb3 integrin expression. By employing curcumin-loaded cyclic RGDyK conjugated exosomes (cRGD-Exo-cur), the researchers were able to significantly reduce the inflammatory response and cellular death in the locations of lesions in

the ischaemic brain of mice. Curcuma longa is the source of curcumin (368 Da), commonly referred to as diferuloylmethane, a polyphenol [86]. Because of its antipathogenic, anti-inflammatory, and antioxidant properties, the medication is used to treat a number of illnesses, including as cancer, metabolic syndrome, neurodegenerative diseases, chronic inflammation, and liver disease [87]. The team also investigated the impact of conjugating the exosomes with the cRGD peptide on the systems' ability to target the ischaemic areas of the mice's brains. The findings showed that the localisation of cRGD-Exo-cur in the tested animal's brain had an ipsilateral/contralateral ratio of 19. However, the fluorescence signal intensity was also increased in other organs, such as the liver and lungs, even though the rise in signal intensity was greater in the lesions' locations. The literature claims that a high expression of αvβ3 integrins caused the drug's greater localisation in the liver, whilst cRGD-Exo's bigger size in the lungs compared to unmodified exosomes was the reason of this [88].

V. STIMULI-RESPONSIVE PEPTIDES

The capacity of DDSs to change the configuration in reaction to certain triggers is known as the authors' stimuli-responsiveness. It is a crucial characteristic of smart DDSs that enables them to operate precisely and regulately to lessen the severity of side effects and improve the therapeutic effectiveness of medications. The characteristics of DDSs may be dramatically altered by various stimulating agents, such as pH, light, magnetic fields, and enzymes, which can alter their internalisation, drug release, size shrinkage, and cell membrane permeability [91]. Photothermal, magnetic, electric, or ultrasonic effects from the outside or local environmental parameters like pH, temperature, redox status, and the concentration of certain molecules (such O2, urea, or enzymes) can manually activate stimuli-responsive systems. [92,93].

5.1. Enzyme-Responsive Peptides

Enzyme-responsive peptides are the most commonly reported among environment-responsive peptides, and the enzymes selected for action seem to be peptidases (or proteases/or proteinases). Enzymes known as peptidases, which are members of the hydrolase class, use water to break covalent peptide bonds (>C(=O)NH-R) [94]. Numerous peptidases have been shown to accumulate more often at lesion locations, including ischaemic and tumour areas. The peptidase classes can be separated into aspartic (cathepsin D, cathepsin E, memapsin), metallo- (gelatinases, matrilysins), cysteine- (cathepsin B, cathepsin C), serine- (uPA, PSA, thrombin), and threonine- (testes-specific protease 50, threonine aspartase 1) [95]. As a result, scientists are now interested in creating biocompatible peptidase-responsive DDSs by using these characteristics of tumour tissues.

In the United States, pancreatic ductal adenocarcinoma (PDAC) is now responsible for 25% of cancer-related fatalities; according to research, this percentage is expected to rise to 50% by 2030. Its resistance to immunotherapy and chemotherapy makes treatment difficult [96]. The primary treatment option for metastatic PDAC is gemcitabine (263 Da), a deoxycytidine nucleoside analogue that achieves its anti-proliferative effect by blocking cell cycle progression at the

G1/S-phase barrier [98]. Gemcitabine prolongs survival for around two to three months, although it nearly always comes with the development of chemo-resistance [99]. One strategy to increase medication efficiency is the use of smart DDSs. Although many different peptidase-responsive peptides are employed in DDSs, a small number have drawn special attention because of their exceptional effectiveness (Figure 1). One such peptide is the GFLG (Gly-Phe-Leu-Gly) tetrapeptide, which is broken by the cysteine protease cathepsin B, which is expressed at greater levels in most tumour types than in normal tissue [100]. The use of GFLG in a DDS has been documented by Zhang and associates. Through cathepsin B-cleavable GFLG, they created a system that depicts PEGylated lysine dendrimer nanoparticles associated with gemcitabine [101]. The dendrimer is very water-soluble and biodegradable due to its construction from the amino acid residue. Together with the dendrimer's branching structure, PEGylation enhances the system's solubility and reduces immunogenicity while extending the drug's blood circulation duration, allowing for less frequent dosing. When cathepsin B cleaves GFLG, the DDS is intended to release gemcitabine. Research has demonstrated that the cathepsin B environment (in vitro) exhibits a gemcitabine release that is over 80% more than that of the control environment. The system's high biocompatibility and the effectiveness of GFLG in particular are demonstrated by the nanoparticles' overall relative tumour suppression volume of 82 ± 38% in a 4T1 murine breast cancer model, which exhibited no symptoms of damage to normal cells. The drug release kinetics showed that 90% of the loaded gemcitabine was released in 24 hours, whereas 60% was released in 30 minutes (Table 2). [101].

Table 2. GFLG conjugated drug delivery systems.

Delivery Vehicle/Targeting Agent	Drug	Mechanism of Action of the Drug	Cancer Type	Outcome	References
PEGylated lysine dendrimer nanoparticles	Gemcitabine	Antiproliferative properties through the blockage of cell cycle progression	Breast cancer	89.9% tumor growth inhibition	[101]
Copper sulfide nanoparticles	DOX	Generation of ROS, inhibition of topoisomerase II, disruption of gene expression	Lymphoma	49.6 ± 1.3% apoptosis rate	[102]
mPEGylated dendron	DOX	Generation of ROS, inhibition of topoisomerase II, disruption of gene expression	Breast cancer	31% apoptotic rate	[107]
CREKA (Cys–Arg–Glu–Lys–Ala) peptide Fibronectin–targeting	Squaraine photosensitizer	Induction of cytotoxicity through the generation of ROS	Triple negative breast cancer	83.5 ± 8.7% tumor inhibition rate	[108]
SKAAKN (Cys–Lys–Ala–Ala–Lys–Asn) peptide	Daunomycin	Topoisomerase II poison, generation of ROS, DNA impairment	Pancreatic ductal adenocarcinoma	0.1 ± 0.1% cell viability	[109]

Daunomycin, a different chemotherapy to DOX and a member of the anthracycline family, has several adverse effects. The medication is used to treat acute lymphoblastic or myeloblastic leukaemia and was initially isolated from Streptomyces peucetius [110]. Numerous mechanisms, such as topoisomerase II poisoning, ROS production, DNA impairment, and the dissociation of H1.1 linker histones from DNA, which results in the degradation of higher-order chromatin structure, facilitate the drug's anticancer impact [111].

A DDS has been proposed by Dokus and associates to deliver GFLG-conjugated daunomycin to the areas of pancreatic cancer. Cell viability decreased in PANC-1 tumor-bearing mice used in in vivo experiments [109].

5.2. pH-Responsive Peptides

Protease cleavage is not the only characteristic of peptides that may be utilised to create stimuli-responsive DDSs. Peptides can react to pH variations since they are composed of charged components. Given that certain clinical diseases, including ischaemia, arthritis, atherosclerosis, and tumours, are known to have reduced pH at the sites of lesion, this

characteristic can be particularly helpful for DDSs [120]. A class of pH-low insertion peptides (pHLIP) is notable among the many known pH-sensitive peptides because of its capacity to cross membranes [121]. It is now well known how pHLIPs work: at physiological pH, pHLIPs have a coiled conformation, are negatively charged, and are hydrophilic, meaning they cannot pass through the plasma membrane. However, pHLIP's C terminus and negative residues become protonated in an acidic environment, which results in a more neutral, lipophilic state and a conformational change from a coiled to an alpha helix. This allows the protein to penetrate the plasma membrane through its neutral C terminus and anchor to it as a transmembrane protein [122,123].

A 36 amino acid long pHLIP peptide is responsible for the pH-sensitive characteristics of the pH-and thermoresponsive gold nanocages (pPGNCs) that Huang and colleagues disclosed [124]. Conversely, poly(di(ethylene glycol) methyl ether methacrylate-co-oligo(ethylene glycol) methyl methacrylate) (PEGDMA-MMA) is credited with thermosensitivity, which reacts to the temperature rise caused by NIR irradiation. The gel's temperature-induced collapse causes the nanocages' pores to open, releasing the drug molecules within. Compared to free DOX, DOX-loaded pPGNCs showed almost five times higher accumulation in tumours and nearly two times lower accumulation in the heart. In contrast to the free DOX, the system also showed a two-fold increase in the drug's liver accumulation. The pH-responsive cell-penetrating capabilities of the pHLIP peptide were confirmed when it was demonstrated that at pH 6.5, a much higher amount of DOX was internalised into the tumour cells (one and a half times more for MCF-7 cells and four times more for Adriamycin-resistant MCF-7 cells) than at pH 7.2. DOX-loaded pPGNCs under light irradiation demonstrated a 97% tumour growth suppression in in vivo anticancer tests on drug-resistant MCF-7 tumor-bearing mice (Table 3) [124]. Han and colleagues created the delivery of gemcitabine via pHLIP-conjugated iron-based nanoparticles that are 35 amino acids long, and they were able to accomplish a 91% tumour growth suppression in 30 days of therapy [125]. Furthermore, it was revealed that 38 amino acid long pHLIP membrane-penetrative properties may be used to transport peptide nucleic acids as big as 7 kDa [126]. Through the production of useful proteins and the RNA interference-induced degradation of mRNA, nucleic acids alter the signalling pathways of cancer cells [127]. Therefore, this strategy may broaden the range of medications that may be used in chemotherapy [126].

Table 3. pHLIP conjugated DDSs.

Delivery Vehicle/Targeting Agent	Drug	Mechanism of Action of the Drug	Cancer Type	Outcome	References
Gold nanocages	DOX	Generation of ROS, inhibition of topoisomerase II, disruption of gene expression.	Breast cancer	97% tumor growth inhibition	[124]
PEGylated Fe_3O_4 nanoparticles	Gemcitabine	Antiproliferative properties through the blockage of cell cycle progression	Pancreatic ductal adenocarcinoma	91.2% tumor growth inhibition after 30 days of treatment	[125]
38 amino acid long	Peptide nucleic acids	Synthesis of functional proteins and the degradation of mRNA through RNA interference	Melanoma	7 kDa peptide translocation	[126]

VI. PEPTIDE-BASED SELF-ASSEMBLY SCAFFOLDS

One intriguing strategy for developing new treatments in regenerative medicine is the production of biomaterials made by short peptides or their derivatives self-assembling. Animal models have shown the remarkable signalling potential and therapeutic efficacy of peptide scaffolds. Many kinds of self-assembling peptide-based scaffolds exist, including hairpin peptides, Fmoc-di and tri peptides, self-

complementary ionic peptides, and peptide amphiphiles. Folding a polypeptide sequence to create tertiary structures is an example of self-assembly, an entropy-driven process that occurs in nature. They may be made to improve cell signalling capacities (growth factors, RNA, DNA, etc.) and to adopt bioactive signalling schemes [128]. Furthermore, peptides with certain characteristics and a controlled degree of breakdown can be used to create more complex and hierarchical structures by manipulations with tiny building blocks. These days, using machine learning to create peptide sequences with specific characteristics is a significant strategy. Amphiphilic peptides may self-assemble into hydrophilic and hydrophobic domain-containing nanostructures. It can have two, three, or four blocks that offer novel functional and structural characteristics as well as affinities for interacting with intracellular organelles or cellular membranes. The human nuclear Ki-67 protein, which functions as a biosurfactant and offers a steric and electrostatic charge barrier against the collapse of mitotic chromosomes, was used to create amphiphilic peptide scaffolds [129].

Although there are several triggers that might cause a simultaneous self-assembly process, the most frequent one is a pH shift that causes the equilibrium to shift from charged to uncharged groups, giving hydrophobic interactions the upper hand. Since transglutaminage, urease, or gamma gluconolacton are typically used, a straightforward pH change with an alkali or acid does not result in the formation of appropriate homogenous scaffolds. Using a self-assembly technique, a macroporous scaffold was created under a variety of circumstances, including extremely low temperatures. Tightly packed nanofibers make up FmocPhePhe-based scaffolds, which have pore sizes ranging from 50 to 150 µm. These scaffolds may be utilised to load hydrophobic medications and cultivate mammalian cells [130]. The capacity to use a concentration below 1%, which is helpful economically, is another benefit of employing peptides for scaffold synthesis. The self-assembly of 9-fluorenylmethoxycarbonyl (Fmoc)-(Leu)n-Gln-Gly to nanofibers was investigated by Wakabayashi and associates. Following the in-situ hydrogel synthesis, the transglutaminase-catalyzed enzymatic process fused the enhanced green fluorescent protein with the MRHKGS tag and connected it to the functional groups of fibres. This method demonstrates the possibility of scaffold modification using growth factors or signalling molecules that are covalently linked [131]. Pandit and associates investigated how the tetrapeptide Boc-Trp-Leu-TrpLeu-OMe self-assembles into spherical nano-/microspheres after dissolving in ethanol. Anti-parallel β sheets were created by hydrogen bonding peptide strands, where the leucine side chains directed towards one side of the β sheet and the tryptophan ring pointed towards the other. It is noteworthy that the nanospheres were produced at a concentration of 0.02 mM and that the hydrophobic effect acted as the mediating agent rather than the aromatic p-p stacking of tryptophan rings. This system's benefit is that, unlike what was seen at 0.156 mM, nanocarriers did not assemble because of the low concentration (0.02 mM). These nanospheres were used to effectively immobilise curcumin [132].

VII. ONGOING/RECENTLY COMPLETED CLINICAL TRIALS

As was previously mentioned, preclinical research showed that peptide-based DDSs were effective against a range of cancer types. The therapeutic benefits of various peptide drug conjugates, such as ANG1005, CBX-12, melflufen, and bicycle peptides (BT5528 and BT8009), are now being studied in a number of clinical studies.

The effectiveness, safety, and tolerability of ANG1005, a novel taxane derivative made up of three paclitaxel molecules covalently bonded to a 19-amino acid Angiopep-2 peptide, were evaluated in 72 adult patients with detectable recurrent brain metastases from breast cancer (BCBM), with or without leptomeningeal carcinomatosis, in an open-label, multicenter phase II study. Angiopep-2 peptides were created to engage with the LRP1 transport mechanism and penetrate the blood–brain barrier (BBB). 86% of patients saw extracranial benefit and 77% of patients experienced intracranial benefit (stable illness or better). Furthermore, intracranial disease control was attained in 79% of patients with leptomeningeal carcinomatosis, and the estimated median overall survival was 8.0 months (95% CI, 5.4–9.4) (NCT02048059) [150]. These findings demonstrated that ANG1005 has the ability to cross the blood-brain barrier and transport paclitaxel to the central nervous system, where it can demonstrate its anticancer properties. The efficacy of ANG1005 in improving patient survival in comparison to a Physician Best Choice control is currently being assessed in 150 HER2-negative breast cancer patients with newly diagnosed leptomeningeal disease and previously treated brain metastases (NCT03613181) through an open-label phase III (ANGLeD) study that has not yet begun recruiting.

Exatecan, a topoisomerase inhibitor coupled to the pH-Low Insertion Peptide (pHLIP)-based platform CBX-12, prevents DNA supercoiling during transcription, replication, and chromatin remodelling by inhibiting the topoisomerase enzymes [151]. In addition, pHLIP selectively targets the tumor's low pH environment without the need for an antigen, enabling the peptide to be inserted into the cancer cell membrane and then released into the tumour cell via glutathione reduction of the linker [152]. For a phase I/II openlabel, multicenter, dose-escalation, safety trial of CBX-12 on 112 patients with advanced or metastatic refractory solid tumours, Cybrexa Therapeutics is now accepting applications. The study's primary outcome measures include assessing the overall response rate, recommended dosage, and incidence of treatment-emergent adverse events (NCT04902872).

Melflufen is a brand-new peptide-drug combination that targets aminopeptidases to release alkylating chemicals into tumour cells quickly and selectively. The effectiveness of melflufen conjugated to dexamethasone was assessed in 157 patients with relapsed and refractory multiple myeloma (RRMM) in a phase II HORIZON study. In the all-treated group (triple-class-refractory illness, extramedullary disease, and refractory to prior alkylator treatment), the overall response rate to melflufen was 29%, whereas in the tripleclass-refractory group, it was 26%. The average overall survival in the all-treated group was 11.6 months at a median follow-up of 14 months with controllable side effects, and the average response time to the medication was 5.5 months (NCT02963493). [153]. In order to compare the safety and effectiveness of melflufen with dexamethasone vs

pomalidomide plus dexamethasone in 495 patients with RRMM that is resistant to lenalidomide, a bigger randomised, controlled, open-label, phase III OCEAN research was announced. Progress-free survival is the study's main goal, whereas overall response rate, response duration, and overall survival are important secondary endpoints (NCT03151811) [154]. But because of a higher risk of mortality from the treatment, the U.S. FDA is now ordering the maker to halt OCEAN trial enrolment despite its expedited clearance.

Table 4. Ongoing/recently completed clinical trials.

#	Study Title	Disease	Treatment (Intervention)	Estimated Enrollment	Current Status and Phase	Trial Number
1	ANG1005 in Breast Cancer Patients With Recurrent Brain Metastases	Breast Cancer, Brain Metastases	Participants intravenously received ANG1005 up to a maximum of one year, or until disease progression or adverse events	72 participants	Completed, Phase II	NCT02048059
2	ANG1005 in Leptomeningeal Disease From Breast Cancer (ANGLeD)	Leptomeningeal Carcinomatosis, Leptomeningeal Metastases, Brain Metastases, HER2-negative Breast Cancer	Participants intravenously received ANG1005 or active comparator: Physician's Best Choice (capecitabine or eribulin or high-dose intravenous (IV) methotrexate)	150 participants	Not yet recruiting, Phase III	NCT03613181
3	Study of CBX–12 in Subjects With Advanced or Metastatic Refractory Solid Tumors	Solid Tumor Adult, Epithelial Ovarian Cancer, Small Cell Lung Carcinoma	CBX–12 administered on a daily × 5 every 3 weeks schedule or a daily × 3 every 3 weeks schedule in ovarian and small lung cancer cohorts	112 participants	Recruiting, Phase I/II	NCT04902872
4	A Study of Melphalan Flufenamide (Melflufen) in Combination With Dexamethasone in Relapsed Refractory Multiple Myeloma Patients (HORIZON)	Multiple Myeloma	Patients received intravenously 40 mg of melflufen on day 1 of each 28-day cycle and once weekly oral 40 mg of dexamethasone (20 mg in patients older than 75 years)	157 participants	Not yet recruiting, Phase II	NCT02963493
5	A Study of Melphalan Flufenamide (Melflufen)–Dex or Pomalidomide–Dex for RRMM Patients Refractory to Lenalidomide (OCEAN)	Multiple Myeloma	Patients received intravenously 40 mg of melflufen on day 1 of each 28-day cycle and once weekly oral 40 mg of dexamethasone or Pomalidomide 4 mg orally daily on days 1 to 21 and dexamethasone 40 mg once weekly of each 28-day cycle	495 participants	Active, not recruiting, Phase III	NCT03151811
6	Study BT5528–100 in Patients With Advanced Solid Tumors Associated With EphA2 Expression	Advanced Solid Tumor Identified as Positive for EphA2 Tumor Expression by Central Laboratory (Phase I), Non Small Cell Lung Cancer Identified as Positive for EphA2 Tumor Expression by Central Laboratory (Phase II)	Patients receive intravenous infusion of BT5528 once a week alone or with nivolumab on a 4-week cycle at the selected dose	166 participants	Recruiting, Phase 1/II	NCT04180371
7	Study BT8009–100 in Subjects With Nectin-4 Expressing Advanced Solid Tumors Malignancies	Advanced Solid Tumor, Urinary Bladder Neoplasm, Pancreatic Neoplasms, Triple Negative Breast Neoplasms, Carcinoma, Non-Small-Cell Lung, Stomach Neoplasm, Esophageal Neoplasms, Ovarian Neoplasm	Patients receive intravenous infusion of BT8009 once weekly alone or with nivolumab on a 4-week cycle at the selected dose	146 participants	Recruiting, Phase 1/II	NCT04561362

VIII. CONCLUSIONS

Peptide-based drug delivery systems (DDS) represent an innovative and promising approach in the field of pharmaceutical drug delivery, offering significant advantages in terms of targeted therapy, enhanced drug stability, and reduced systemic side effects. The design and synthesis of peptide-drug conjugates have shown that peptides can effectively target specific receptors or tissues, facilitating the precise delivery of therapeutic agents to disease sites. This approach has the potential to revolutionize treatments, particularly in complex conditions such as cancer, autoimmune diseases, and chronic inflammatory disorders.

The successful development of peptide-based DDS requires a careful balance between the peptide's ability to target specific cells and tissues, its stability within the body, and the controlled release of the drug. Advances in peptide synthesis techniques, such as solid-phase peptide synthesis (SPPS), and conjugation methods, including click chemistry and enzyme-mediated conjugation, have enabled the production of stable and efficient peptide-drug conjugates. Furthermore, in vitro evaluations, including drug release kinetics, cytotoxicity, and receptor binding studies, have provided valuable insights into the performance of these DDS, demonstrating their potential for clinical applications.

However, challenges such as enzymatic degradation, stability in physiological conditions, and large-scale manufacturing remain. To overcome these challenges, ongoing research is focusing on improving peptide stability, enhancing targeting efficiency, and optimizing the release profiles of peptide-based DDS. The integration of peptide-based DDS with other drug delivery systems, such as nanoparticles or liposomes, also holds great promise in further enhancing their efficacy.

In conclusion, peptide-based drug delivery systems hold immense potential for the

future of medicine, offering new avenues for targeted therapies with reduced adverse effects. As research progresses and these systems are optimized, peptide-based DDS may become a cornerstone of personalized and precision medicine, improving patient outcomes and transforming the treatment of various diseases.

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