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Therapeutic Peptides Encapsulated into Lipid-Based Nanovesicles: Scaling from Lab to Plant

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Abstract - Lipid-based peptide-encapsulated nanovesicles represent a cutting-edge approach in drug delivery systems, offering enhanced stability, targeted delivery, and controlled release of therapeutic peptides. This review explores the comprehensive science and techniques behind the robust formulation and analytical methods for these nanovesicles, spanning from lab-scale research to large-scale manufacturing. Critical aspects include lipid selection, detailing how the choice of lipids impacts the stability and efficacy of the nanovesicles, and various peptide encapsulation techniques, emphasizing methods to achieve high encapsulation efficiency and controlled release. Key processes in vesicle formation, such as thin film hydration, sonication, and microfluidics, are discussed to highlight their roles in producing nanovesicles with consistent size and morphology. The importance of stability studies, including physical, chemical, and thermal assessments, is underscored. Analytical techniques such as Dynamic Light Scattering (DLS), Zeta Potential measurement, High-Performance Liquid Chromatography (HPLC), and Mass Spectrometry (MS) are detailed, showcasing their crucial roles in characterizing nanovesicles and ensuring their quality, safety, and efficacy for pharmaceutical use. The review addresses the challenges and solutions associated with scaling up production, including maintaining consistent product quality, optimizing manufacturing processes, and controlling production costs. It also navigates the regulatory landscape, discussing the stringent guidelines set by agencies such as the FDA and EMA and the necessity of strategic regulatory planning to facilitate market approval. This comprehensive review aims to serve as an in-depth resource for experts in the field, aiding in the translation of lipid-based peptide-encapsulated nanovesicle formulations from bench to bedside, ultimately enhancing therapeutic outcomes and expanding treatment options for various disease.

Key Words: Peptide, Amino acid, Degradation, Pharmacokinetics, Peptide encapsulation, drug delivery systems, Peptide Characterization

1. INTRODUCTION

Therapeutic peptides, comprising short sequences of amino acids, have emerged as promising candidates for treating a variety of diseases by mimicking the functions of natural proteins. Their inherent specificity and potency afford them several advantages over traditional small-molecule drugs, particularly in terms of targeting and minimizing off-target effects. Peptides can modulate a range of biological processes, making them valuable in the treatment of conditions such as cancer, diabetes, cardiovascular diseases, and infectious diseases (1)(2). Their ability to precisely interact with biological targets enables them to offer therapeutic benefits that are often difficult to achieve with small molecules.

However, despite their considerable therapeutic potential, peptides face significant challenges related to bioavailability. These challenges include enzymatic degradation, poor membrane permeability, rapid clearance, and immunogenicity. Enzymatic degradation is a major concern, as peptides are susceptible to breakdown by proteolytic enzymes within the gastrointestinal tract and bloodstream, which diminishes their therapeutic efficacy (3). Poor membrane permeability further compounds this issue, as the hydrophilic nature and larger size of peptides limit their ability to cross cellular membranes effectively (4,5). Rapid clearance through renal filtration also necessitates frequent dosing, which can be inconvenient and reduce patient compliance. Additionally, some peptides may elicit immune responses, leading to reduced efficacy and potential safety concerns (6).

To address these bioavailability issues, lipid-based formulations have emerged as a resourceful and efficient approach. Lipid-based nanovesicles, such as liposomes, Niosomes solid lipid nanoparticles (SLNs), and nanostructured lipid carriers (NLCs), provide several key advantages by enhancing peptide stability, protecting against enzymatic degradation, and improving membrane permeability (7,8). Encapsulation within these nanovesicles shields peptides from harsh physiological conditions, thereby preserving their therapeutic activity and improving their solubility. The lipid matrix of these vesicles acts as a barrier, preventing enzymatic breakdown and facilitating the controlled release of peptides. In addition, lipid-based formulations, as well as novel approaches by using lipid synthesis inhibitors (LSIs), cell-penetrating peptides (CPPs), and ionic liquids (ILs), can be specifically engineered to gain permeability and target particular cells or tissues, thereby amplifying the therapeutic potential of peptides, while simultaneously minimizing off-target effects and decreasing systemic toxicity. (9,10)

Among the advantages of lipid-based nanovesicles are enhanced stability, improved bioavailability, targeted delivery, and controlled release. Encapsulation within lipid-based nanovesicles protects peptides from other routes of degradation and hydrolysis, addressing common challenges in peptide drug delivery (11). By incorporating peptides into lipid matrices, these formulations can significantly improve peptide absorption through biological membranes, thus increasing bioavailability. Targeted delivery capabilities are achieved by modifying the lipid composition or surface characteristics of the vesicles, which allows for precise targeting of therapeutic peptides to specific cells or tissues. Furthermore, the lipid matrix can be designed to provide sustained or controlled release of encapsulated peptides, reducing the need for frequent dosing and enhancing overall therapeutic efficacy.

Natural flavonoids loaded vesicular systems are useful in treatment of lung cancer. (7,8,9,10,11,12).

Despite these benefits, scaling up the production of lipid-based peptide-encapsulated nanovesicles from lab-scale formulations to industrial-scale manufacturing presents several challenges. Consistency and quality control are critical, as variations in lipid composition, peptide loading, and manufacturing processes can impact the stability, size distribution, and release characteristics of nanovesicles. Scalability requires significant adjustments to processes such as lipid mixing, vesicle formation, and size reduction to handle larger volumes while maintaining product quality (13,14). Compliance with regulations introduces additional complexity, as it is essential to meet strict guidelines established by organizations like the FDA and EMA. This includes tox studies, defined preclinical studies, clinical studies demonstrating safety, efficacy, and quality, and ensuring compliance with Good Manufacturing Practices (GMP) (15,16).

This review article will provide a brief understanding on the chemistry, therapeutic area and pharmacokinetic challenges of therapeutic peptides, a detailed examination of the science and techniques involved in the development and characterization of lipid-based peptide-encapsulated nanovesicles, explore lipid selection and formulation, discuss the properties of phospholipids, cholesterol, and synthetic lipids, and their influence on nanovesicle formation and stability. Peptide encapsulation techniques, including passive and active methods, will be examined to understand how to achieve high encapsulation efficiency and controlled peptide release. Various vesicle formation techniques, such as thin film hydration, sonication, extrusion, and microfluidics, will be reviewed alongside stability studies that assess physical, chemical, and thermal stability. Analytical methods, including dynamic light scattering (DLS), zeta potential measurement, high-performance liquid chromatography (HPLC), and mass spectrometry (MS), will be described to evaluate the size, charge, encapsulation efficiency, and stability of nanovesicles. Finally, we will discuss the challenges associated with scaling up production, including process optimization, equipment selection, and cost considerations, as well as strategies for maintaining quality control and regulatory compliance during large-scale manufacturing.

2. Therapeutic Peptides: Chemistry, Therapeutic Areas, and Pharmacokinetic Challenges

Therapeutic peptides have garnered significant attention due to their unique ability to interact with biological systems with high specificity and potency. These short sequences of amino acids offer a promising alternative to traditional small-molecule drugs such as higher specificity, reduced off-target effects, and the potential for addressing previously intractable therapeutic targets. However, their clinical efficacy is often limited by pharmacokinetic challenges that affect their stability, bioavailability, and overall therapeutic potential (1,2,17).

2.1. Chemistry of Therapeutic Peptides

The chemistry of therapeutic peptides is crucial in understanding their function, stability, and effectiveness. Peptides usually consist of 2 to 50 amino acids connected by peptide bonds, creating linear or cyclic structures. A peptide's biological activity and stability are determined by its amino acids' sequence and composition. For therapeutic applications, peptides can be designed to mimic natural hormones, neurotransmitters, or other bioactive molecules (18).

2.1.1. Structure and Design:

Therapeutic peptides are pharmaceutical agents made up of a series of amino acids, usually with a molecular weight of 500–5000 Da. They are flexible molecules that can mimic protein's local structural features, such as hydrophobicity, secondary structure, and electrostatic charge distribution. However, peptides generally don't have a defined three-dimensional structure, unlike proteins which have a defined tertiary and quaternary structure. Some peptides can have a defined three-dimensional structure due to the presence of hydrogen bonds, hydrophobic interactions, and multiple disulfide bridges (2,19, 20).

2.1.2. Synthesis and Modification:

Peptide synthesis is typically achieved through solid-phase peptide synthesis (SPPS) or liquid-phase synthesis. Solid-phase peptide synthesis (SPPS) is a common method for producing synthetic peptides in a lab and is considered a major tool for peptide synthesis. It's more efficient than liquid-phase peptide synthesis (LPPS) because it allows for easier product isolation. SPPS involves adding protected amino acid derivatives to a growing peptide chain that's anchored to an insoluble polymer. The process includes washing and deprotection steps to remove side products and unreacted groups (21). Post-synthetic modifications, such as cyclization, acetylation, or PEGylation, can further enhance the peptide's stability and pharmacokinetic properties (22). Deep generative models (DGMs) have emerged as tools for designing therapeutic peptides. These tools include generative adversarial networks (GANs), variational autoencoder (VAE), and diffusion models, which can help generate novel peptide sequences that meet specific objectives. However, automated or assisted peptide design still faces challenges, such as developing and validating predictive models, establishing informative representation strategies, and optimizing peptide processing.

2.2. Therapeutic Areas and Examples

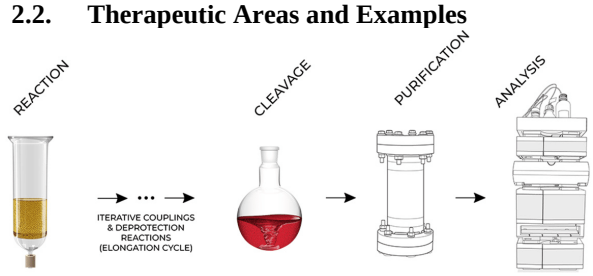

Fig -1: Process of solid-phase peptide synthesis (SPPS)

Therapeutic peptides have been successfully applied in various clinical areas, showcasing their versatility and potential.

2.2.1. Cancer Therapy:

Cancer is the second leading cause of death worldwide and one of the most serious health issues (8). Traditional chemotherapy remains a first-line treatment, with drugs such as Cyclophosphamide, Cisplatin, Carmustine, and Bendamustine commonly employed for cancer therapy (23,24). However, these drugs are associated with significant toxic effects. Recent advancements in cancer treatment focus on more targeted approaches, such as the use of peptides that specifically target tumor antigens or growth factors.

Peptide-based therapies have emerged as a promising option in cancer treatment. For example, the peptide medication bivalirudin is used as an anticoagulant in patients

undergoing percutaneous coronary interventions. Another notable peptide, somatostatin, is effective in treating neuroendocrine tumors by inhibiting hormone secretion, offering a more tailored treatment approach compared to traditional chemotherapy (25).

Recent advances in immunotherapy have transformed cancer treatment by enhancing the immune system's ability to target malignant cells, especially in advanced-stage cancers. This review, covering research from 1985 to the present, explores key inhibitory and stimulatory immune checkpoint pathways tested in preclinical models and clinical trials. Findings suggest that combination therapies targeting multiple pathways could maximize treatment effectiveness while reducing toxicity, paving the way for immunotherapy to complement traditional cancer treatments as a promising strategy (26).

I. Bivalirudin: An Anticoagulant in Cancer Therapy

Bivalirudin is a synthetic peptide drug that acts as a direct thrombin inhibitor. It is primarily used as an anticoagulant in patients undergoing percutaneous coronary interventions (PCI), a procedure commonly performed to treat coronary artery disease. Although not directly used for cancer treatment, its role as an anticoagulant can be crucial for cancer patients undergoing various medical procedures where blood clot prevention is necessary. Bivalirudin's effectiveness in such scenarios underscores the broader utility of peptide-based therapies in supporting cancer patients' overall treatment plans.

II. Somatostatin: Inhibiting Hormone Secretion in Neuroendocrine Tumors

Somatostatin, a cyclic peptide hormone, controls the endocrine system and impacts neurotransmission and cell growth by hindering the release of multiple secondary hormones. In the treatment of cancer, synthetic versions of somatostatin, like octreotide and lanreotide, are administered to address neuroendocrine tumors. These tumors frequently produce an abundance of hormones, resulting in diverse clinical manifestations. Somatostatin and its analogs help manage these symptoms by inhibiting hormone secretion, thus improving patients' quality of life and potentially slowing tumor growth (25).

By targeting specific pathways and mechanisms involved in cancer, peptide drugs like bivalirudin and somatostatin demonstrate the versatility and potential of peptide-based therapies in oncology. These examples highlight how peptides can be tailored to address various aspects of cancer treatment, from anticoagulation to hormone regulation, ultimately contributing to more effective and comprehensive care for cancer patients.

2.2.2. Diabetes Management: Peptide Hormones

Diabetes management often relies on the administration of peptide hormones to regulate blood glucose levels. Insulin is a hormone made by the pancreas in the form of a peptide, and it is crucial for cells to absorb glucose. Administering external insulin is a fundamental part of the treatment for people with diabetes, especially those with Type 1 diabetes and some with Type 2 diabetes (27). Insulin analogs, such as insulin lispro and insulin glargine, have been developed to improve glycemic control by offering pharmacokinetic profiles that better mimic the body's natural insulin response compared to regular human insulin (26,28).

I. Insulin Lispro: Rapid-Acting Insulin Analog

Insulin lispro is a rapid-acting insulin analog designed to mimic the body's natural postprandial (after meal) insulin response. The onset of action is quicker, and the duration is shorter compared to regular human insulin, which means it is very good at managing blood sugar spikes following meals. Insulin lispro is typically administered shortly before meals to manage postprandial hyperglycemia (26,28,29).

II. Insulin Glargine: Long-Acting Insulin Analog

Insulin glargine is a long-acting insulin analog designed to provide a steady and prolonged release of insulin, maintaining basal insulin levels for an extended period. This steady release reduces the risk of nocturnal hypoglycemia and helps maintain consistent blood glucose levels throughout the day and night. The usual administration schedule for insulin glargine is once a day, which offers a more convenient and efficient basal insulin treatment for patients (26, 28,30).

III. Pharmacokinetic Profiles

The pharmacokinetic profiles of insulin lispro and insulin glargine offer significant advantages over regular human insulin. Regular insulin has a slower onset and longer duration of action, which can lead to suboptimal postprandial glucose control and an increased risk of hypoglycemia. In contrast, insulin lispro's rapid onset allows for timely glucose uptake following meals, while insulin glargine's prolonged action provides a stable basal insulin level, minimizing fluctuations in blood glucose (28).

The development of insulin analogs like insulin lispro and insulin glargine represents a significant advancement in diabetes management. By enhancing glycemic control and reducing the risk of hypoglycemia, these analogs improve the living standards of people with diabetes. Their improved pharmacokinetic profiles provide more flexibility and precision in insulin therapy, aligning more closely with the body's natural insulin response and facilitating better overall diabetes management.

2.2.3. Cardiovascular Diseases: Peptide-Based Therapies

In recent trends, the chromene nucleus has emerged as a significant scaffold for the development of new drug candidates, owing to its diverse pharmacological activities such as antitumor, anti-inflammatory, and antiviral properties. Traditional methods for synthesizing chromene derivatives, however, often involve hazardous reagents and generate toxic waste, raising environmental concerns. To mitigate these issues, green chemistry approaches have been introduced, utilizing sustainable raw materials, non-toxic catalysts, and milder reaction conditions to reduce ecological impact.

Meanwhile, peptides have gained considerable attention as a safer alternative in therapeutic interventions, particularly in cardiology. B-type natriuretic peptide (BNP) and angiotensin II receptor antagonists are notable examples of peptide-based treatments currently used in clinical practice to effectively diagnose and manage conditions like heart failure and hypertension (31,32,33).

I. B-type Natriuretic Peptide (BNP): Diagnosing and Managing Heart Failure

The hormone B-type natriuretic peptide (BNP) is generated by the heart as a reaction to ventricular volume expansion and pressure overload, which are frequently observed in heart failure. Increased levels of BNP in the bloodstream are a sign of heart failure and are linked to the seriousness of the condition. BNP measurements are used

both diagnostically and prognostically to guide the management of heart failure. (34,35)

a. Diagnostic Value of B-type Natriuretic Peptide (BNP)

BNP testing is particularly valuable in the emergency department setting, where rapid and accurate diagnosis of heart failure is critical. High BNP levels can confirm the diagnosis of heart failure, helping to differentiate it from other causes of dyspnea (shortness of breath). Additionally, BNP levels provide prognostic information, with higher levels indicating more severe heart failure and a worse prognosis. (34,35,36)

b. Therapeutic Monitoring

BNP levels are also used to monitor the effectiveness of heart failure treatment. Decreasing BNP levels over time generally indicate an improvement in heart failure status, while increasing levels may signal worsening heart failure or the need for therapy adjustments. (37)

Peptide-based therapies, such as BNP for diagnosing and managing heart failure and ARBs for treating hypertension and heart failure, play crucial roles in cardiovascular medicine. By providing valuable diagnostic information and effective treatment options, these peptide-based interventions contribute significantly to improving patient outcomes in cardiovascular diseases.

2.2.4. Infectious Diseases: Antimicrobial Peptides (AMPs)

Antimicrobial peptides (AMPs) are a diverse group of molecules that form a critical part of the innate immune system. They possess wide-ranging antimicrobial effects against bacteria, fungi, viruses, and even certain parasites. AMPs have attracted interest as possible treatments, particularly in light of increasing antibiotic resistance. One notable example of AMPs is defensins, which have demonstrated efficacy against a wide range of bacterial and fungal pathogens (38).

I. Antimicrobial Peptides (AMPs)

AMPs are typically small, positively charged peptides that have the ability to disrupt the structure of microbial cell membranes, ultimately causing the cells to die. Their mechanism of action generally involves the attraction to and integration into negatively charged microbial membranes, causing membrane disruption and subsequent cell lysis. This mode of action is different from traditional antibiotics, which makes AMPs less likely to induce resistance. (38)

II. Defensins: A Key Example of AMPs

Defensins are a family of AMPs found in many organisms, including humans. They are categorized into alpha, beta, and theta defensins based on their structure and the cells that produce them. Human defensins, particularly human alpha-defensins and beta-defensins, play a significant role in the first line of defense against infections (39,40).

III. Efficacy Against Bacterial Pathogens

Defensins have shown potent activity against a wide range of bacterial pathogens, including both Gram-positive and Gram-negative bacteria. For example, human beta-defensin 2 (hBD-2) exhibits effective antimicrobial action against Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa. These peptides work by binding to the bacterial membrane and forming pores, this disrupts the membrane and results in the death of bacterial cells. (39,40)

IV. Efficacy Against Fungal Pathogens

In addition to their antibacterial properties, defensins are effective against fungal pathogens. For instance, human beta-defensin 3 (hBD-3) has demonstrated activity against Candida albicans, a common fungal pathogen. This activity is crucial for the management of fungal infections, which are difficult to treat. (39,40)

V. Therapeutic Potential of AMPs

Given their broad-spectrum activity and unique mechanism of action, AMPs like defensins are being explored as potential therapeutic agents. Their ability to target a wide array of pathogens while reducing the likelihood of resistance development makes them attractive candidates for new antimicrobial therapies (38,39,40).

Antimicrobial peptides, particularly defensins, represent a promising class of therapeutic agents with broad-spectrum antimicrobial activity. Their efficacy against various bacterial and fungal pathogens, combined with their unique mechanism of action, highlights their potential in the fight against infectious diseases, especially in an era of increasing antibiotic resistance.

Table-1: Therapeutic Peptides their indication and target receptor approved by USFDA(2)

Peptide Name	*USFDA approved Indication(s)*	*Target Receptor Name*	*Year First approval*
Aviptadil	Treatment of erectile dysfunction	VIP1 receptor	2000
Atosiban	Indicated for use in delaying imminent pre-term birth	OT receptor	2000
Taltirelin	Spinocerebellar degeneration	TRH receptor	2000
Carbetocin	Used for postpartum hemorrhage	OT receptor	2001
Nesiritide	Treatment of acute decompensated heart failure	NPR-A	2001
Teriparatide	Treatment of osteoporosis	PTH1 receptor	2002
Abarelix	Treatment of advanced prostate cancer	GnRH receptor	2003
Enfuvirtide	Used in combination therapy for the treatment of HIV-1	gp41	2003
Ziconotide	Management of severe chronic pain	N-type calcium channels	2004
Exenatide	Indicated for Type 2 Diabetes Mellitus	GLP-1 receptor	2005
Pramlintide	Treatment of Type 1 and Type 2 Diabetes Mellitus	Calcitonin receptor	2005
Degarelix	Treatment of advanced prostate cancer	GnRH receptor	2008
Icatibant	Approved for use in acute attacks of hereditary angioedema	Beta2-receptor	2008
Romiplostim	Treatment of chronic immune thrombocytopenic purpura	Thrombopoietin receptor	2008
Liraglutide	Indicated for Type 2 Diabetes Mellitus	GLP-1 receptor	2009
Mifamurtide	Treatment of high-grade, resectable, non-metastatic osteosarcoma	NOD2 protein	2009
Tesamorelin	Reduction of HIV lipodystrophy	GHRH receptor	2010
Teduglutide	Treatment of Short bowel syndrome and malabsorption	GLP-2 receptor	2012
Linaclotide	Treatment of irritable bowel syndrome (IBS) with constipation and chronic idiopathic constipation	GC-C receptor	2012
Carfilzomib	Treatment of multiple myeloma	Binding to active site of the 20S proteasome	2012
Peginesatide	Managing anemia linked to long-term kidney disease.	Human erythropoietin receptor	2012
Lucinactant	Prevention of respiratory distress syndrome	Pulmonary surfactant	2012
Pasireotide	Treatment of Cushing's disease	Somatostatin receptors	2012
Lixisenatide	Indicated for Type 2 Diabetes Mellitus	GLP-1 receptor	2013
Albiglutide	Indicated for Type 2 Diabetes Mellitus	GLP-1 receptor	2014
Dulaglutide	Indicated for Type 2 Diabetes Mellitus	GLP-1 receptor	2014
Afamelanotide	Prevention of phototoxicity	MC1 receptor	2014
Etelcalcetide	Indicated for secondary hyperparathyroidism	CaSR	2016
Semaglutide	Indicated for Type 2 Diabetes Mellitus	GLP-1 receptor	2017
Abaloparatide	Treatment of osteoporosis	PTH1 receptor	2017
Plecanatide	Treatment of chronic idiopathic constipation	Guanylate cyclase C	2017
Angiotensin II	Indicated for sepsis and septic Shock	AT_1 receptor	2017
Lutetium Lu 177 dotatate	Treatment of somatostatin receptor-positive gastroenteropancreatic neuroendocrine tumors	Somatostatin receptors	2018
Bremelanotide	Indicated for hypoactive sexual desire disorder	MC receptors	2019
Edotreotide gallium Ga-68	Indicated for diagnose somatostatin receptor positive neuroendocrine tumors	Somatostatin receptors	2019
Setmelanotide	Indicated for chronic weight management of obesity	Melanocortin-4 receptor	2020

Table-2: Therapeutic Peptides under investigation in clinical studies(2)

Therapeutic Peptide name	Indication(s) for investigation	Clinical study-Phase
Avexitide	Hypoglycemia	IV
Calcitonin gene-related peptide	Migraine	IV
Corticorelin	Brain swelling; brain neoplasms	IV
Leptin	Lipodystrophy; obesity	IV
Thymalfasin	Liver Cirrhosis; Sepsis	IV
Aclerastide	Diabetic foot ulcers	III
Albusomatropin	Growth hormone deficiency	III
Anamorelin	Cachexia; lung cancer non-small cell cancer	III
G17DT	Various forms of cancer	III
Insulin peglispro	Diabetes mellitus	III
Lenomorelin	Malignancies	III
Selepressin	Shock; septic	III
Somapacitan	Adult growth hormone deficiency	III
Taspoglutide	Type 2 diabetes mellitus	III
Thymosin beta-4	Dry eye syndrome	III
Tirzepatide	Type 2 diabetes mellitus	III
Ularitide	Decompensated heart failure	III
Vapreotide	Gastric varices; oesophageal haemorrhage; portal hypertension; esophageal varices	III
Vosoritide	Achondroplasia	III
Zoptarelin doxorubicin	Endometrial cancer; prostate cancer	III
Angiotensin 1-7	Miscellaneous Peripheral Blood Cell Abnormalities	II
Bombesin	Prostate cancer	II
Cenderitide	Heart failure	II
Deslorelin	Puberty; precocious	II
Gastric inhibitory polypeptide	Type 2 diabetes mellitus	II
MK-3207	Migraine	II
Olcegepant	Migraine Disorders	II
Pancreatic Polypeptide	Type 1 diabetes	II
Peptide YY (3-36)	Metabolic disease; obesity	II
Pirnabine	Chronic idiopathic constipation	II
Somatopri	Acromegaly	II
Somatropin pegol	Growth hormone deficiency	II
Thyrotropin	Benign nontoxic and toxic goiter; goiter; nodular	II
TT-232	Renal cell adenocarcinoma	II
BPI-3016	Type 2 diabetes mellitus	I
NBI-6024	Type 1 diabetes mellitus	I

2.3. Pharmacokinetic Challenges

Despite their therapeutic promise, peptides face several pharmacokinetic challenges that limit their clinical use. These challenges include enzymatic degradation, poor membrane permeability, rapid clearance, and immunogenicity (41).

2.3.1. Enzymatic Degradation and controlling strategies:

Peptides can be broken down or degraded by proteolytic enzymes found in the digestive system (GI tract) and in the blood. This enzymatic degradation is a significant barrier to their effective therapeutic use, particularly for oral administration. The primary enzymes responsible for peptide breakdown include pepsin in the stomach and trypsin and chymotrypsin in the small intestine. Once peptides are degraded into their constituent amino acids, their therapeutic efficacy is lost. This challenge necessitates the development of protective delivery systems or peptide modifications to enhance their stability and bioavailability (41, 42).

2.3.1.1. Mechanisms of Enzymatic Degradation

In the GI tract, the acidic environment of the stomach and the presence of digestive enzymes contribute to the rapid degradation of peptides. Proteolytic enzymes cleave peptide bonds, resulting in the breakdown of the peptide into inactive fragments. (41,42)

A. Strategies to Prevent Degradation

I. Protective Delivery Systems

Numerous delivery systems have been investigated to safeguard peptides from enzymatic degradation. These systems include:

i. Lipid Nanoparticles and Liposomal preparation:

Lipid nanoparticles (LNPs) have gained significant attention in the pharmaceutical industry as effective carriers for delivering a range of therapeutic agents. Their use has also expanded into various sectors such as medical imaging, cosmetics, nutrition, agriculture, and innovative applications like nanoreactors. The first iteration of lipid nanoparticles, liposomes, are highly versatile and capable of transporting both lipid-soluble and water-soluble drugs. By encapsulating these medications, liposomes provide protection against rapid degradation and reduce toxicity by minimizing systemic exposure. They can also improve the therapeutic efficacy of both new and existing drugs by altering pharmacokinetic parameters like absorption and metabolism, leading to a prolonged biological half-life and slower elimination. With hydrophilic inner and outer surfaces and a hydrophobic middle layer, liposomes are adept at encapsulating a wide variety of drugs and targeting them to specific sites. This functionality has been exploited in the formulation of numerous anticancer drugs, including those currently on the market, to achieve precise and targeted delivery. Advancements in LNP technology have led to the development of next-generation particles such as solid lipid nanoparticles, nanostructured lipid carriers, and cationic lipid–nucleic acid complexes, which feature more intricate internal structures and greater physical stability (43,44,45,46).

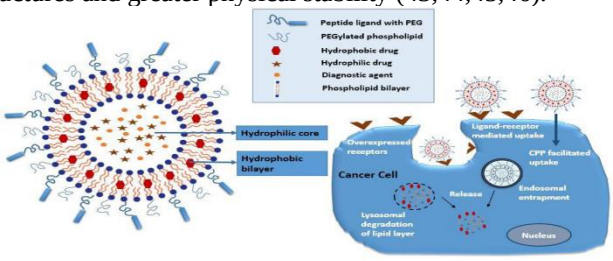

Fig – 2: Schematic presentation of functionalized peptide-targeted liposomes drug delivery mechanism

ii. Hydrogels:

Hydrogels, which are cross-linked polymer networks, can encapsulate peptides, offering a protective shield against proteolytic enzymes. These hydrogels can be precisely engineered to release peptides at targeted locations. In recent years, peptide-based hydrogel systems have garnered significant attention for various healthcare applications, such as drug delivery platforms, topical antimicrobial agents, tissue engineering scaffolds, and wound healing (47,48). This popularity stems from the unique chemical and functional versatility of peptides, which can be tailored to self-assemble into supramolecular hydrogels in response to physiological stimuli, including pH changes, salt concentrations, and the presence of specific enzymes. Peptides can be modified at the molecular level, allowing for the fine-tuning of properties like hydrogel formation, mechanical strength, sustained drug release, and antimicrobial effectiveness (47,49,50).

Peptide hydrogels are often preferred over synthetic polymeric systems due to their enhanced biocompatibility and biodegradability, making them promising candidates for novel biomedical technologies. However, the approach does have limitations. Naturally occurring L-α enantiomers of peptide amino acids are more easily recognized by proteolytic enzymes, leading to rapid degradation and clearance in vivo, which diminishes their effectiveness as long-acting drug delivery platforms (51). To address this issue, researchers have explored non-natural peptide-like molecules, known as peptide-mimetics, to enhance the biostability and bioavailability of native peptides (52). These modifications involve altering the chemical structure of amino acids to create non-native peptide analogues, such as D-amino acids (53), β-amino acids (54), γ-amino acids (55), and peptoids (56). The clinical potential of peptide-mimetic hydrogels for long-acting drug delivery is exemplified by degarelix (Firmagon), a hormonal therapy for advanced castration-

sensitive prostate cancer. Degarelix is a synthetic peptide-mimetic comprising ten amino acids, five of which are D-amino acids, providing both hydrogel-forming ability and active therapeutic properties (46,57).

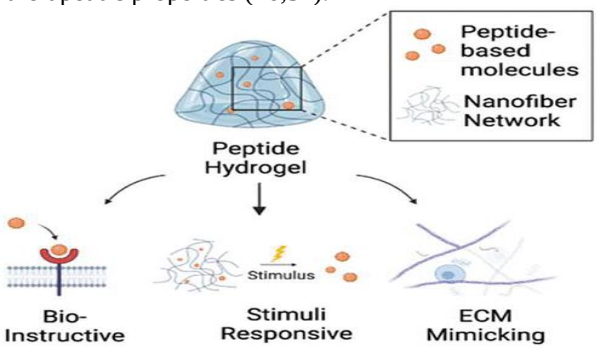

Fig – 3: Hydrogel delivery system to encapsulate the peptides (47)

B. Chemical Modifications

Peptide chemical modifications are crucial in enhancing the stability, bioavailability, and functionality of peptides used in various therapeutic and biomedical applications. Key methods of peptide chemical modifications include acetylation, cyclization, N-methylation, and covalent modifications (58,59,60)

I. Acetylation

Acetylation involves the addition of an acetyl group to the N-terminus or lysine residues of a peptide. This modification can increase peptide stability by protecting against enzymatic degradation and can improve membrane permeability, thus enhancing its pharmacokinetic profile. For example, acetylation has been shown to significantly enhance the therapeutic efficacy of peptide drugs by prolonging their half-life and reducing immunogenicity (61).

II. Cyclization

Cyclization, the process of forming a cyclic structure in a peptide, can dramatically improve its stability and binding affinity. Cyclized peptides are less susceptible to proteolytic enzymes due to the rigid structure, which can also enhance receptor binding specificity and potency. Methods such as head-to-tail cyclization and side-chain-to-side-chain cyclization have been employed to develop more stable and potent peptide therapeutics (62).

III. N-methylation

N-methylation involves the addition of a methyl group to the nitrogen atom of the peptide backbone. This modification can increase the metabolic stability and oral bioavailability of peptides by making them less recognizable to proteolytic enzymes. N-methylation also improves the ability of peptides to cross cellular membranes, which is beneficial for intracellular targets (63).

IV. Covalent modifications

Covalent modifications, including the attachment of polyethylene glycol (PEGylation), glycosylation, and lipidation, can enhance the pharmacokinetic and pharmacodynamic properties of peptides. PEGylation, for example, increases the molecular size of peptides, reducing renal clearance and extending their circulation time in the body (64). Glycosylation can improve peptide solubility and stability, while lipidation enhances membrane affinity and cell penetration (65).

Chemical modifications such as acetylation, cyclization, N-methylation, and covalent modifications are essential strategies for optimizing peptide therapeutics. These modifications enhance peptide stability, bioavailability, and functional properties, making them more effective for various clinical applications.

2.3.2. Poor Membrane Permeability

The poor membrane permeability of therapeutic peptides presents a major obstacle in drug delivery, limiting their clinical efficacy. This issue stems from several inherent characteristics of peptides. Firstly, their relatively large molecular size and hydrophilic nature hinder their ability to pass through the lipid-rich cellular membranes. Secondly, peptides are prone to enzymatic degradation in the gastrointestinal tract and bloodstream, which further reduces their bioavailability(67,68).

To address these challenges, several strategies have been developed to enhance the membrane permeability of therapeutic peptides. One effective approach involves the use of cell-penetrating peptides (CPPs). CPPs are short peptides that can traverse cell membranes and facilitate the internalization of therapeutic peptides into cells. By conjugating CPPs to therapeutic peptides, their cellular uptake can be significantly improved (67,68).

Chemical modification techniques also play a crucial role in enhancing peptide permeability. For instance, lipidation, which involves attaching lipid moieties to peptides, increases their lipophilicity, allowing better interaction with cell membranes. Cyclization, another chemical modification, stabilizes peptide structures, making them less susceptible to enzymatic degradation and improving their membrane permeability (58,59,60,61).

Nanoparticle-based delivery systems represent another promising strategy. Encapsulating therapeutic peptides in nanoparticles protects them from enzymatic degradation and enhances their stability. These nanoparticles can also be engineered to target specific cells or tissues, thereby improving the efficiency of peptide delivery (43,44,45,46,47,49,50,69).

Additionally, the use of permeation enhancers can temporarily disrupt the cell membrane, allowing peptides to pass through more easily. Designing prodrugs, where the therapeutic peptide is chemically modified to improve its properties and then converted back to the active form within the body, can also help in overcoming permeability issues (67,69,70).

By leveraging these strategies, the bioavailability and therapeutic efficacy of peptide-based drugs can be significantly improved, paving the way for their successful application in various medical treatments.

2.3.3. Rapid Clearance

Therapeutic peptides, despite their promising therapeutic potential, face significant pharmacokinetic challenges, particularly regarding renal clearance. The kidneys efficiently filter these small, hydrophilic molecules, leading to rapid excretion in the urine. This swift renal clearance often results in a short half-life and necessitates frequent dosing to maintain therapeutic plasma concentrations, which can limit the clinical utility of peptide-based therapies. Understanding and addressing these challenges is crucial for the successful development of peptide therapeutics (71,72).

One widely adopted strategy to mitigate rapid renal clearance is PEGylation, the process of attaching polyethylene glycol (PEG) chains to the peptide molecule. PEGylation increases the hydrodynamic size of the peptide, thereby reducing its glomerular filtration rate (GFR) and subsequent

renal excretion. Additionally, PEGylation can enhance the peptide's solubility and stability, protecting it from enzymatic degradation and immune recognition. As a result, PEGylated peptides exhibit prolonged circulation times and improved pharmacokinetic profiles, making them more effective for therapeutic use(64,65,73).

Another effective approach involves the use of albumin-binding peptides or the creation of fusion proteins. Albumin is a naturally abundant plasma protein with a long half-life of approximately 19 days. By designing peptides that bind to albumin or by genetically fusing therapeutic peptides to albumin or albumin-binding domains, the half-life of the peptide can be significantly extended. This strategy exploits the inherent longevity of albumin in the bloodstream, reducing the frequency of dosing and enhancing therapeutic efficacy (73,74).

Incorporating non-natural amino acids or D-amino acids into peptide sequences is another strategy to combat rapid renal clearance (75). Peptides composed of L-amino acids are susceptible to proteolytic enzymes, leading to rapid degradation and clearance. However, peptides with non-natural or D-amino acids exhibit increased resistance to enzymatic breakdown, resulting in reduced renal clearance and prolonged plasma retention. This approach not only improves the pharmacokinetic properties but also enhances the overall stability and bioavailability of the therapeutic peptides (75).

Lipidation, the attachment of lipid moieties to peptides, is also employed to improve pharmacokinetics. Lipidated peptides exhibit increased plasma protein binding, particularly to albumin and lipoproteins, which reduces their renal filtration and extends their circulation time. Additionally, lipidation can facilitate the targeting of peptides to specific tissues or cells, enhancing their therapeutic potential while minimizing systemic exposure and side effects (76,77).

In conclusion, addressing the pharmacokinetic challenges related to renal clearance is essential for optimizing the therapeutic potential of peptide-based drugs. Strategies such as PEGylation, albumin binding, incorporation of non-natural amino acids, lipidation, and prodrug design have shown considerable promise in enhancing the pharmacokinetic profiles of therapeutic peptides. By implementing these strategies, researchers can develop more effective and longer-lasting peptide therapies, ultimately improving patient outcomes and expanding the clinical applications of these versatile molecules.

2.3.4. Immunogenicity

Therapeutic peptides hold great promise for treating a range of diseases due to their specificity, potency, and generally favorable safety profiles. However, their clinical application is often impeded by pharmacokinetic challenges related to immunogenicity. Immunogenicity refers to the capacity of a substance to provoke an immune response, which, in the case of therapeutic peptides, can lead to the production of anti-drug antibodies (ADAs). These antibodies can neutralize the therapeutic effect of peptides, alter their pharmacokinetics, and cause adverse immune reactions.

One significant pharmacokinetic challenge posed by immunogenicity is the rapid clearance of therapeutic peptides from the bloodstream. When ADAs are generated, they can form immune complexes with the therapeutic peptides, which are then quickly removed via the reticuloendothelial system (RES), reducing the drug's half-life and diminishing its therapeutic window. For instance, patients receiving therapeutic peptides like interferons often develop ADAs, resulting in a notable decrease in drug efficacy, necessitating higher doses or more frequent administrations (78,79).

To overcome these challenges, several strategies have been devised. One effective approach is modifying the peptide structure to reduce its immunogenic potential. Techniques such as PEGylation, where polyethylene glycol (PEG) chains are attached to the peptide, can shield the epitopes recognized by the immune system, thus reducing immunogenicity. PEGylation has been successfully applied to several therapeutic peptides, enhancing their stability and extending their half-life (64,65,73).

Advanced delivery systems also play a crucial role in overcoming immunogenicity-related pharmacokinetic challenges. Liposomes, nanoparticles, and other carrier systems can encapsulate therapeutic peptides, protecting them from the immune system and enzymatic degradation. These carriers can be engineered to release the peptide in a controlled manner, ensuring sustained therapeutic levels and reducing the frequency of administration (43,44,45,46,47,49,50,69).

Another strategy involves the use of immunosuppressive therapies alongside therapeutic peptides. Co-administration of immunosuppressive agents, such as corticosteroids or methotrexate, can help mitigate the immune response against the peptides. This approach has been particularly effective in patients receiving biologic therapies, where immunogenicity is a significant concern (80).

Moreover, advances in bioengineering have enabled the development of peptide analogs with reduced immunogenicity. By altering specific amino acids or incorporating non-natural amino acids, it is possible to create peptides that retain their therapeutic activity but are less likely to be recognized by the immune system. Such modifications can significantly improve the pharmacokinetic profile of therapeutic peptides (75).

In conclusion, while immunogenicity presents significant pharmacokinetic challenges for therapeutic peptides, a combination of structural modifications, immunosuppressive co-therapies, advanced delivery systems, and bioengineering innovations offers promising solutions. By addressing these challenges, the clinical efficacy and safety of therapeutic peptides can be greatly enhanced, paving the way for their broader application in treating various diseases.

Therapeutic peptides offer a promising approach to treating a wide range of diseases due to their specificity and potency. However, their clinical application is limited by pharmacokinetic challenges, including enzymatic degradation, poor membrane permeability, rapid clearance, and immunogenicity. Advancements in lipid-based formulations, chemical modifications, controlled release systems, and targeted delivery strategies are crucial for overcoming these challenges and enhancing the therapeutic potential of peptides. Continued research and development are essential for translating the promise of therapeutic peptides into effective clinical treatments.

3. Lipid-Based Encapsulated Peptides: Formulation Development and Technological Advancements

The therapeutic potential of peptides is often constrained by their intrinsic pharmacokinetic and stability issues. Peptides, despite their high specificity and potency, suffer

from rapid enzymatic degradation, poor membrane permeability, and rapid clearance from the body (1). Lipid-based encapsulation methods have emerged as a promising approach to address these challenges. By incorporating peptides into lipid-based nanocarriers, researchers have improved the stability, bioavailability, and controlled release of peptide drugs (2).

3.1. Chemistry of Lipid-Based Encapsulation

Lipid-based nanocarriers are designed to encapsulate peptides within lipid matrices, providing a protective environment that enhances the therapeutic potential of the peptides (44,45,46,81). These formulations are classified based on their structure and composition, including liposomes, solid lipid nanoparticles (SLNs), and nanostructured lipid carriers (NLCs) (82).

3.1.1. Liposomes

Liposomes are spherical vesicles composed of one or more phospholipid bilayers surrounding an aqueous core. They are one of the earliest and most studied lipid-based carriers for peptide drugs. The liposomal bilayer acts as a barrier, protecting peptides from enzymatic degradation and facilitating their controlled release. Liposomes can be tailored to enhance peptide stability and release properties by varying the lipid composition and bilayer structure (44,45,46,81).

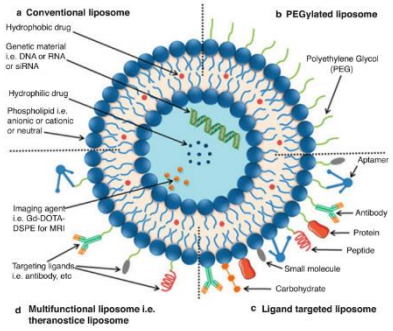

Fig – 4: Schematic illustration of Liposomal structure of therapeutic peptides. (81)

3.1.2. Solid Lipid Nanoparticles (SLNs)

SLNs are composed of a solid lipid matrix that encapsulates the peptide drug. Unlike liposomes, SLNs are solid at body temperature, which provides additional stability to the encapsulated peptides (48). The solid lipid matrix protects peptides from oxidation and enzymatic degradation, while also allowing for sustained release. The choice of solid lipid materials and the formulation process significantly influence the properties of SLNs (82.83).

3.1.3. Nanostructured Lipid Carriers (NLCs)

NLCs combine the features of SLNs and liquid lipid-based systems. They consist of a solid lipid matrix with a fraction of liquid lipid, creating a more flexible and versatile carrier system. NLCs offer enhanced drug loading capacity and stability compared to SLNs. The dual lipid matrix in NLCs helps to improve the encapsulation efficiency and control the release of peptide drugs (82,83).

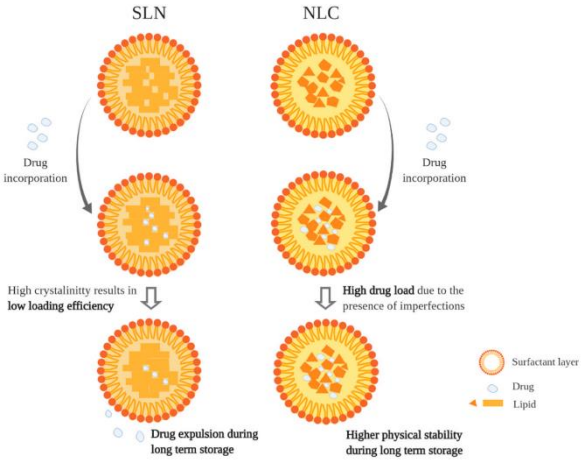

Fig – 5: Diagrammatic Representation of Solid Lipid Nanoparticles and Nanostructured Lipid Carriers (82)

3.2. Key Components and Formulation Techniques

The development of lipid-based peptide formulations involves several key techniques to ensure optimal encapsulation, stability, and release profiles.

3.2.1. Lipid Selection

The choice of lipids is critical in formulation development. Phospholipids, such as phosphatidylcholine and phosphatidylserine, are commonly used due to their biocompatibility and ability to form bilayers. Cholesterol is often added to enhance membrane rigidity and stability. Synthetic lipids can also be employed to tailor the properties of lipid-based carriers (84,85).

3.2.2. Peptide Encapsulation Methods

Peptide encapsulation is crucial for enhancing the stability, bioavailability, and controlled release of peptide drugs. Encapsulation can be achieved through passive or active methods, each with specific techniques and applications (85,86,87,88,89).

Table-3: Peptide encapsulation methods

Encapsulation technique	***Passive encapsulation***			***Active encapsulation***		
Description	Passive encapsulation involves incorporating peptides into lipid-based carriers during their formation. This method typically results in the entrapment of peptides within the carrier matrix without requiring additional steps post-formation.			Active encapsulation involves loading peptides into pre-formed lipid-based carriers using specific techniques. This approach often provides better control over peptide loading and release profiles.		
Encapsulation method	Thin-Film Hydration Method(87)	Sonication(87)	High-Pressure Homogenization(87)	Reverse Phase Evaporation(88)	pH Gradient Method(89)	Microfluidic Technology(90)
Process	Lipids are dissolved in an organic solvent and evaporated to form a thin lipid film. This film is then hydrated with an aqueous solution containing peptides, forming liposomes or lipid nanoparticles.	n aqueous solution of lipids and peptides is subjected to ultrasonic waves, which induce the formation of nanometric vesicles.	This technique involves passing a lipid and peptide mixture through a high-pressure homogenizer to create nanoparticles or liposomes.	A mixture of lipids and peptides is dissolved in an organic solvent and then evaporated to form a lipid film. The film is hydrated in a peptide solution, resulting in the formation of vesicles with encapsulated peptides.	Lipid-based carriers are formed in a pH gradient environment, where the peptide is loaded into the carriers due to the pH difference between the interior and exterior of the carriers.	Lipid and peptide solutions are mixed using microfluidic devices, which precisely control the mixing conditions to form nanoparticles or liposomes with encapsulated peptides.
Advantages	Simple and cost-	Allows to produce	Produces nanoparticles	Allows for high	Provides a high	Allows for precise

Encapsulation technique	Passive encapsulation			Active encapsulation		
	effective, suitable for large-scale production.	liposomes with high encapsulation efficiency.	with a narrow size distribution and can be scaled up for industrial applications.	peptide encapsulation efficiency and control over the size and stability of the carriers.	encapsulation efficiency and is useful for loading peptides that are sensitive to pH changes.	control over particle size and encapsulation efficiency, suitable for high-throughput screening.

3.3. Scalability Challenges and Future Aspects for Encapsulated Peptide Formulation

Encapsulated peptide formulations hold great promise for improving the stability, bioavailability, and targeted delivery of therapeutic peptides. However, scaling up these formulations from laboratory research to commercial production presents significant challenges (91).

One of the primary scalability challenges is the reproducibility of particle size and distribution. During the scale-up process, maintaining consistent particle size is crucial for ensuring uniformity in drug release profiles and therapeutic efficacy. Variations in the manufacturing process, such as changes in mixing speeds, temperatures, and solvent evaporation rates, can lead to inconsistencies in particle size distribution, impacting the final product's quality and performance (91).

Another challenge lies in the formulation's stability during the production and storage phases. Peptides are inherently unstable and prone to degradation, aggregation, or denaturation, which can be exacerbated by the conditions of large-scale manufacturing. Strategies to enhance stability, such as optimizing the lipid composition, incorporating stabilizing excipients, or employing advanced encapsulation techniques, are crucial for successful scale-up (59,92).

The choice of encapsulation method also influences scalability. Techniques such as solvent evaporation, hot melt extrusion, and spray drying each have their unique challenges and limitations when scaled up. For example, solvent evaporation requires careful control of solvent removal to prevent peptide degradation (85), while hot melt extrusion must manage thermal stability concerns (93). Adapting these methods to large-scale production while maintaining product quality is a complex task.

Regulatory compliance and quality control present additional hurdles. Ensuring that the scaled-up process meets regulatory standards for safety, efficacy, and quality requires thorough validation and robust quality control measures. This includes rigorous testing for encapsulation efficiency, drug loading, release profiles, and stability, which can be resource-intensive and time-consuming.

Looking to the future, advancements in encapsulation technologies and manufacturing processes are likely to address many of these challenges. Innovations such as microfluidics, continuous manufacturing, and automated process control offer potential solutions for improving the scalability of encapsulated peptide formulations. Additionally, ongoing research into novel excipients and delivery systems may provide new avenues for enhancing stability and bioavailability on a commercial scale.

In conclusion, while there are significant challenges in scaling up encapsulated peptide formulations, continued research and technological advancements hold promise for overcoming these barriers. By addressing issues related to particle size consistency, stability, encapsulation methods, and regulatory compliance, the future of peptide-based therapeutics looks promising.

4. Analytical Methodology for Characterization and Regulatory Expectations

Lipid-based nanovesicles have revolutionized drug delivery systems by providing efficient means to encapsulate and deliver therapeutic peptides. These nanovesicles offer significant advantages, including enhanced stability, improved bioavailability, and targeted delivery of peptides. To ensure their efficacy and safety, rigorous analytical characterization is essential.

4.1. Analytical Methodologies for Characterization

Characterizing lipid-based peptide nanovesicles involves a range of analytical techniques to assess their physicochemical properties, stability, and performance.

Table-4: Quality attributes and methods for characterization (94)

Quality attributes	Method and parameters
Size and Size Distribution	Transmission electron microscopy (TEM) and scanning electron microscopy (SEM)
Surface Charge (Zeta Potential)	DLS, Electrophoretic mobility
Encapsulation Efficiency (%)	HPLC, UV/VIS, Fluorescence
Lamellarity	Freeze-fracture electron microscopy and small-angle X-ray scattering (SAXS)
In vitro Drug Release	Dialysis methods, diffusion cells, and sample-and-separate techniques
Peptide Integrity	Mass Spectrometry, Circular Dichroism (CD) Spectroscopy:
Stability	Parameters such as temperature, pH, and ionic strength, Size and size distribution, drug release

4.1.1. Particle Size and Size Distribution

Particle size and size distribution are critical parameters influencing the stability and efficacy of lipid-based nanovesicles. Techniques such as Dynamic Light Scattering (DLS) and Nanoparticle Tracking Analysis (NTA) are commonly used for this purpose. DLS measures the intensity of scattered light from particles in suspension to determine their size and distribution, while NTA visualizes and tracks individual particles to provide size and concentration data (94,95).

4.1.2. Zeta Potential

The zeta potential is an indicator of the surface charge of nanovesicles and is crucial for assessing their stability in suspension. Techniques like Electrophoretic Light Scattering (ELS) are used to measure zeta potential, which reflects the repulsive forces between particles and can predict their tendency to aggregate. A high absolute zeta potential typically correlates with better stability and reduced aggregation (94,96).

4.1.3. Encapsulation Efficiency

Encapsulation efficiency refers to the percentage of peptide that is successfully incorporated into the nanovesicles compared to the total amount used in the formulation. Methods such as High-Performance Liquid Chromatography (HPLC) and Spectrophotometry are employed to measure encapsulation efficiency. HPLC provides detailed separation and quantification of peptides, while spectrophotometric methods use absorbance or fluorescence to estimate peptide concentration (94)

4.1.4. Release Kinetics

The release kinetics of peptides from lipid-based nanovesicles are assessed to understand their therapeutic behavior. Techniques such as In Vitro Release Studies and Mathematical Modeling are used to evaluate how peptides are released over time under physiological conditions. Release studies typically involve sampling the release medium at

various time points and quantifying the amount of peptide released (97).

4.1.5. Stability Studies

Stability studies are essential to ensure that lipid-based nanovesicles retain their properties over time. Techniques include Physical Stability Tests, such as freeze-thaw cycles and accelerated stability testing, and Chemical Stability Tests, which assess the degradation of peptides within the nanovesicles. These studies play a crucial role in determining the shelf life and optimal storage conditions for the formulations.

4.1.6. Morphological Analysis

Morphological analysis provides insights into the shape and structure of nanovesicles. Techniques such as Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) are used to visualize the nanovesicles at high resolution (94). TEM offers detailed images of internal structures, while SEM provides surface morphology information.

4.1.7. Peptide Lipid interactions

Differential Scanning Calorimetry (DSC) is commonly used to investigate the interactions between membrane-active peptides (e.g., antimicrobial, cell-penetrating) and lipid membranes, which are essential for their biological functions. The binding affinity of peptides to lipid membranes can be determined by the (DSC). It distinguishes between surface adsorption and insertion into the hydrophobic core.

4.2. Encapsulated Peptide Characterization (97)

The following tests are recommended to ensure that the function and structural integrity of encapsulated peptides are maintained during the formulation process.

4.2.1. Primary Structure

In a polypeptide chain, the amino acid sequence is held together by peptide bonds. The form and functions of a protein are determined by its unique fundamental structure. To determine the primary structure two approaches may be applied i.e. bottom up and top-down approach. Bottom-up approach involves the Peptide mapping followed by identification of digested fragments using the Liquid chromatograph attached with the UV and MS detector. While the Top-down approach involves the Tandem mass spectrometry.

4.2.2. Secondary structure and Higher Order Structure [HOS]

The secondary structure of proteins refers to local folded structures within a polypeptide chain, stabilized mainly by hydrogen bonds. The two most common types of secondary structures are the α-helix and the β-pleated sheet. In an α-helix, the carbonyl group of one amino acid forms a hydrogen bond with the amino group of an amino acid four residues down the chain, creating a helical structure. In β-pleated sheets, segments of the polypeptide chain align next to each other, forming a sheet-like structure stabilized by hydrogen bonds between the carbonyl and amino groups of the backbone.

Higher order structure (HOS) encompasses secondary, tertiary, and quaternary structures. The tertiary structure refers to the overall three-dimensional shape of a single protein molecule, determined by interactions between the side chains of amino acids. Quaternary structure involves the arrangement of multiple protein subunits in a multi-subunit complex.

Secondary structure, tertiary structure and HOS can be evaluated by the instruments like Circular dichroism, NMR, XRD, Fluorescence Spectroscopy, IR Spectroscopy

4.2.3. Aggregation/Oligomer/Higher Molecular Weight Impurity

Protein aggregation refers to the process where proteins misfold and self-assemble into oligomers and larger aggregates. It can occur at any stage of the biopharmaceutical process, including bioprocessing, purification, formulation, packaging, and storage. Factors such as light, temperature, pH, shear forces, and interactions with excipients or container materials can induce aggregation. Aggregates can range from small soluble particles to large visible particles and include reversible non-covalent and irreversible covalent bonded species, such as dimers, oligomers, and higher multiples of the protein product (104).

According to regulatory guidelines, accurate quantification of aggregates is required to meet pharmaceutical specifications and understand the nature of protein structure and protein aggregation conditions. Different orthogonal methods are required to assess the aggregation in drug product. HP-SEC (High performance Size Exclusion Chromatography) method is widely used method for quantitative evaluation of peptide aggregates in drug products

5. Conclusion

The robust formulation and analytical method development for lipid-based peptide-encapsulated nanovesicles are pivotal for their successful translation from lab-scale research to large-scale manufacturing in the pharmaceutical industry. This journey begins with the meticulous selection of appropriate lipids, which significantly impact the stability, encapsulation efficiency, and release profile of the nanovesicles. The optimization of encapsulation techniques, whether through passive or active methods, is crucial for achieving high peptide loading and uniform distribution within the vesicles. Additionally, precise control over vesicle formation processes, such as thin film hydration, sonication, and microfluidics, is essential to produce nanovesicles with consistent size and morphology, which are critical parameters for their in vivo performance.

Comprehensive analytical methods are required to thoroughly characterize these formulations, ensuring they meet the stringent quality, safety, and efficacy standards necessary for pharmaceutical applications. Techniques such as Dynamic Light Scattering (DLS), Zeta Potential measurement, High-Performance Liquid Chromatography (HPLC), and Mass Spectrometry (MS) provide detailed insights into the size, surface charge, encapsulation efficiency, and stability of the nanovesicles. These analytical tools are indispensable for quality control, helping to identify and mitigate potential issues that could affect the therapeutic performance of the nanovesicles.

Scaling up production from the lab to an industrial scale presents several formidable challenges. Maintaining product quality across large batches requires stringent process controls and robust quality assurance protocols. Variations in lipid composition, process parameters, and peptide loading can lead to inconsistencies that affect the stability and efficacy of the final product. To address these issues, process optimization is critical, involving the refinement of manufacturing protocols to ensure reproducibility and consistency at larger scales. This includes the adoption of continuous manufacturing processes

and advanced equipment capable of handling increased production volumes without compromising quality.

Regulatory compliance is another significant hurdle. Navigating the complex regulatory landscape involves adhering to guidelines set by agencies such as the FDA and EMA, which require comprehensive documentation and demonstration of safety, efficacy, and quality through rigorous preclinical and clinical testing. Strategic regulatory planning is essential to streamline the approval process, involving early engagement with regulatory bodies and thorough preparation of the necessary documentation to support the regulatory submissions.

Controlling costs is also a major consideration in scaling up production. Efficient use of resources, optimization of manufacturing processes, and leveraging economies of scale are strategies that can help manage production costs. Investment in automation and advanced manufacturing technologies can also enhance efficiency and reduce labor costs, contributing to the overall economic viability of the large-scale production of lipid-based peptide-encapsulated nanovesicles.

Addressing these challenges through a combination of scientific innovation, process optimization, robust quality control, and strategic regulatory planning is essential for the successful commercialization of lipid-based peptide-encapsulated nanovesicles. By overcoming these hurdles, the pharmaceutical industry can harness the full potential of these advanced drug delivery systems, ultimately improving therapeutic outcomes and expanding the range of treatable conditions with peptide-based therapies.

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