CHAPTER 1

INTRODUCTION

"Research has been called good business, a necessity, a gamble, a game. It is none of these- it's a state of mind."

-Martin H. Fischer

1. Introduction

1.1 Peptide Therapeutics

Peptides represent a distinct class of biological substances that create a link between large biologics (usually with a molar mass >5000 g/mol) and small molecules (usually with a molar mass <500 g/mol). Peptides are unique therapeutic agents that hold promise for treating a variety of serious and previously untreatable diseases. Harnessing the unique properties and pharmacological advantages of peptides, these compounds are increasingly recognized as ground breaking drugs and are hailed as promising alternatives to traditional small molecule drugs [1]. Today, peptides are employed in the treatment of a diverse array of health conditions, including complex ailments such as heart disease, various forms of cancer, and a range of metabolic disorders. Moreover, they are emerging as impactful therapeutic agents for respiratory diseases, gastrointestinal issues, urinary tract infections, and infectious diseases, showcasing their versatility and effectiveness.

In comparison to antibody therapies and recombinant proteins, peptide-based treatments stand out for being relatively more affordable while also demonstrating a safer profile with fewer adverse effects. They are less likely to provoke unwanted immune responses and show a significantly reduced tendency to accumulate in the body's tissues, which can be a concern with other biopharmaceuticals. Despite encountering some challenges, such as their higher molecular weight, limited systemic absorption, rapid clearance through hepatic and renal pathways, and low permeability across cell membranes, an impressive number of peptides have successfully transitioned to commercialization. These therapeutics have delivered noteworthy clinical outcomes, proving their efficacy against a wide range of diseases [2]. Since the second half of the 20th century, the market landscape for medicinal and diagnostic peptides has expanded consistently, reflecting their growing significance in healthcare. By the year 2023, over 115 peptide pharmaceuticals had received official approval, marking a remarkable milestone in the advancement of therapeutic options and highlighting the optimism surrounding peptide-based medicine.

At present, more than 60 peptide-based medications are actively available in the pharmaceutical markets of the United States, Japan, and Europe, displaying the growing interest and investment in peptide therapeutics. In addition, over 150 peptides are currently

undergoing rigorous clinical evaluations, with 260 peptides being tested in human trials and more than 400 peptides still in various stages of development, highlighting the robust pipeline of research in this field [3]. In 2023, the global market size for peptide therapeutics was valued at approximately $43.45 billion. This market is projected to experience significant growth, with an anticipated compound annual growth rate (CAGR) of 6.1% through the end of the current decade, which could elevate the market value to around $66.41 billion by 2030 (Report ID: 978-1-68038-179-5) [4].

1.1.1 Peptide therapeutics for addressing unmet needs in healthcare

A substantial amount of data has been collected to reveal that diseases are increasingly recognized as interconnected rather than solitary conditions. However, it is important to note that a variety of diseases emerge from distinct molecular origins, leading to a complex landscape of health issues. As a result, the pressing challenge lies in addressing the phenomenon where even well-known and widely occurring diseases are progressively differentiating into numerous subgroups, each exhibiting unique responses to treatment. This underscores the vital need for the development and implementation of advanced, personalized diagnostic techniques that can accurately capture the intricate realities of these subsets, coupled with swift and effective treatment solutions [5].

Furthermore, the ability to navigate the inherent limitations of human biology by harnessing the potential of innovative delivery technologies plays a crucial role in the effective management of various diseases [6]. Improvements in drug design and product development for peptide-based treatments have significantly changed the average life expectancy and improved quality of life. However, during product development and scale-up, peptide-based treatments would need to undergo some modification, purification, processing, de novo design (utilizes only knowledge about a biological target or its known active binders to create new chemical entities.) and optimization in contrast to small molecular medicines (Figure 1.1). Because of the continuous developments in computational science, the process of designing peptides and their formulations as well as the screening process for peptide-based medicines is progressively growing. The primary aim of the screening procedure is to provide a scientific edge while facilitating a more efficient process for optimizing formulations [7]. A crucial

aspect of this approach involves a detailed examination of the molecular characteristics associated with each ingredient in the formulation. By concurrently integrating chemical, biological, and structural data, this technology proves to be exceptionally valuable in the development of formulations, particularly in scenarios where peptides play a significant role [8].

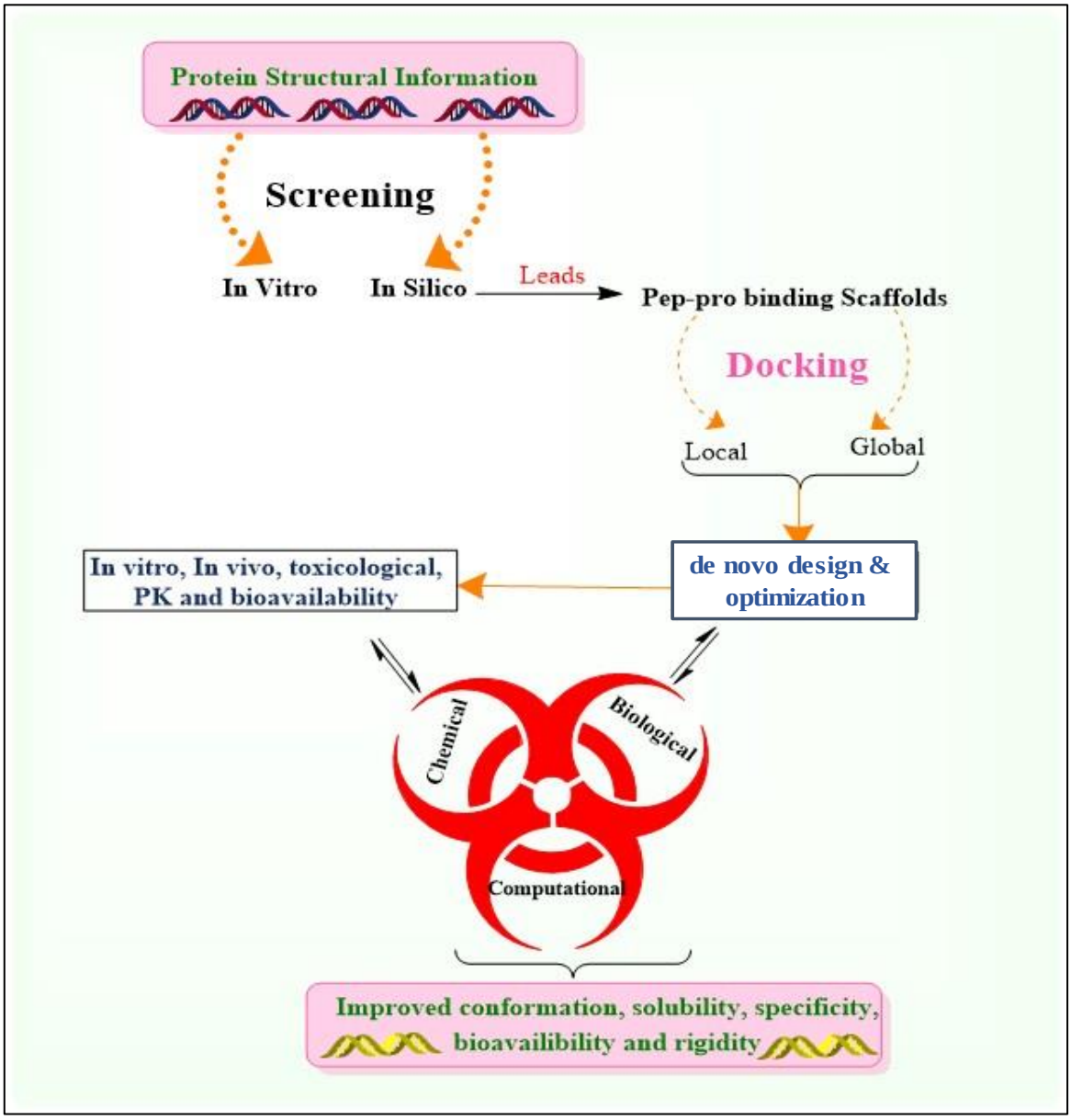

Figure 1.1: A summary of the design process for peptide therapeutics and their associated delivery systems.

Several limiting features that can hinder their therapeutic effectiveness characterize peptide medications. These include a low affinity and selectivity toward their specific biological targets, difficulties in traversing cell membranes due to their solubility challenges, inherent instability when administered within the body, and a notably short half-life in plasma, often resulting from the action of proteases that enzymatically degrade them. Because of these factors, maintaining a drug concentration that is effective for clinical purposes often necessitates frequent dosing regimens.

Moreover, the processes involved in detecting and purifying peptides face significant constraints, primarily due to the relatively small size of these molecules [9]. To address these challenges, innovative chemical modifications of peptides have been developed. This leads to the synthesis of well-defined bioconjugates that exhibit enhanced chemical properties, making them more suitable for both biological studies and the development of novel drug therapies [10]. These modifications aim to prevent degradation by proteolytic enzymes, extend the half-life of the peptides *in vivo*, and ultimately improve their bioavailability and pharmacokinetic characteristics. Various strategies, such as the incorporation of specific amino acids, conjugation with beneficial moieties, cyclization to stabilize the structure, and the addition of lipid components or linkers, have been employed to achieve these goals [11].

Moreover, the process of chemically modifying peptides becomes essential for those that are prone to rapid metabolism, particularly within the liver, and swift elimination through the kidneys. These modifications serve the dual purpose of extending the biological half-life of these peptides while also satisfying the specific imaging requirements associated with radiolabelled peptides. Notably, the presence of various residues of amino acid at either the N- or C-terminal positions can significantly influence the susceptibility of these peptides to proteolysis and degradation, a phenomenon that has been extensively documented in scientific literature.

When the sequences at either the N- or C-terminus are adjusted while still preserving the essential targeting specificity and affinity, these alterations can lead to a decrease in proteolytic degradation, ultimately enhancing the drug's bioavailability in the body [12]. In a similar manner, if such modifications do not hinder the drug's proper functioning, the addition of a C-

terminal amidation or N-terminal acetylation can serve to significantly improve the drug's stability in vivo, allowing for a more effective therapeutic action [13].

Furthermore, the therapeutic peptides' ineffective transport to the site of action is one of their drawbacks. Nanoparticles can be employed to get over this limitation because of their great potential as medication carriers. Therapeutic peptides that have been developed into nanostructures provide the potential to extend their half-life in plasma and accelerate up smart delivery to intended location. These intricate nanostructures, measuring less than one micron in size, are specifically crafted to interact with biological macromolecules and other reactive substances, allowing them to remain stable as colloidal particles suspended in water. Nanoparticles have revealed a remarkable ability to function as innovative conjugate scaffolds or to offer specialized cavities for the entrapment of biologics, thereby significantly enhancing their overall functionality [14]. The quest for developing peptide-derived therapeutic nanomedicines is driven by potential for efficient cellular internalization, along with stable spatial arrangements and improved vascular accessibility offered by nanoparticles, all aimed at overcoming the various pharmacological challenges posed by peptides. Additionally, these nanoparticles can be expertly modified to exhibit stimuli-responsive properties, enabling them to release their therapeutic cargo in response to specific internal or external triggers, thus maximizing their effectiveness in treatment applications [15]. The gradual release of therapeutic peptides from nanoparticles represents an innovative and promising strategy aimed at significantly enhancing their therapeutic effectiveness by reducing the occurrence of off-target effects. This approach includes peptide therapies specifically designed to effectively traverse the blood-brain barrier without the necessity of invasive procedures, which often pose risks to patients. This unique capability opens up new avenues for exploring their potential in the treatment of various neurological disorders and assessing their compatibility with biological membranes at a cellular level. Furthermore, it plays a crucial role in preventing unintended interactions with associated proteins and receptors, especially those that are commonly found circulating within the bloodstream. In addition, the use of drug carriers is proposed as a means to amplify treatment efficacy, while simultaneously controlling the precise release of peptide molecules into the bloodstream to mitigate the toxic effects that can arise from certain peptide compounds [16]. The final critical elements needed to enhance the

speed of the authorization process involve the development of widely accepted clinical testing protocols that ensure consistency and reliability. Additionally, there is a pressing need for detailed regulatory guidelines that encompass all aspects of production, quality assurance, technology standards, and safety measures associated with macromolecular drugs.

In the following sections, we will outline the existing challenges related to certain peptides and investigate potential solutions through the application of advanced formulation techniques and innovative delivery systems.

1.2 Oxytocin

Oxytocin, a nonapeptide composed of nine specific amino acids, serves as both neuropeptide and hormone. It plays a vital role in several physiological processes and is synthesized in the hypothalamus, an essential region of the mammalian brain. This hormone is known for its influence on social bonding, emotional regulation, and reproductive behaviors, making it integral to many aspects of mammalian life. It is used in females to treat incomplete, inevitable or elective abortions, lactation, parturition, and regulate postpartum uterine hemorrhage [17-19].

Within the complex architecture of the brain, oxytocin operates as both neurotransmitter and neuromodulator, playing a crucial role in neural communication. This remarkable hormone acts as a chemical messenger [17] while simultaneously reducing inflammation among neurons, offering a protective barrier against cellular damage [18]. It plays a vital role in shielding the hippocampus an essential region for memory and learning from harmful effects of excitotoxicity, a condition that can lead to cell death in the brain [18-20]. The significance of oxytocin extends into realm of neuropsychiatric and degenerative disorders, where it has shown a promising role in mitigating the symptoms and progression of diseases such as Alzheimer’s disease (AD) [21] and Parkinson’s disease (PD) [22]. At present, oxytocin is offered in different regulatory markets for obstetric and gynecological treatments through intravenous (IV) or intramuscular (IM) administration, with dosages ranging from 5 to 10 units per mL. The challenges of maintaining oxytocin's efficacy in situations where refrigeration temperatures cannot be assured is a frequent cause of concern. The effective dose of oxytocin

may decrease with prolonged exposure to high temperatures. Toxic degradation products may result from the active ingredient's degradation.

Because oxytocin has a short duration of action and is classified as a hydrophilic molecule, only a limited amount of its effective concentration is able to reach the brain when delivered intravenously. Interestingly, numerous clinical and preclinical studies have shown that administering oxytocin through the nasal route significantly improves symptoms related to various neurodegenerative disorders [23]. To tackle the challenges associated with the blood-brain barrier (BBB) entry, researchers have explored a range of innovative technologies in the past, all aimed at enhancing the pharmacological response [24]. Among these strategies are engineered nano drug delivery systems designed specifically to optimize the delivery of oxytocin to the brain. These systems have the potential to minimize toxicity while maximizing therapeutic efficacy within the central nervous system [25].

1.3 Vasopressin

Vasopressin, a nonapeptide is a single linear chain molecule of nine amino acids, produced from enormous precursor proteins (propeptides), which in turn are derived from "prepropeptides" and comes under the class of macromolecules [26]. In the 19^{th} century, vasopressin was initially identified in patients who experienced elevated blood pressure after receiving pituitary extract intravenously. The Vasopressin market is anticipated to grow at a compound annual growth rate (CAGR) of 8.2% throughout the forecast period from 2024 to 2031.Today, vasopressin has recognition in major physiological events from maintenance of arterial pressure to edema in brain to restoration of kidney functions and management of neurological disorders [27,28].

Vasopressin has been demonstrated to be a strong vasoconstrictor in vascular smooth muscle cell (VSMC) as observed in both *in vivo* and cultured cell preparations. Following exposure to the hormone, increase in contractile force are seen within seconds to minutes and are mediated through the V1a receptor [30]. However, current formulations of vasopressin necessitate refrigeration to ensure proper maintenance and reconstitution of lyophilized powders. This requirement arises from the substance's inherent instability over extended periods, making it crucial to store it under specific conditions to preserve its effectiveness [29]. Moreover, it has

a very short half-life (10-35 minutes), being metabolized by vasopressinases. Therefore, certain strategies including nanocarrier-based drug delivery systems were reported to enhance its stability and half-life [25].

1.4 Renin Angiotensin System (RAS)

RAS is essential for preserving electrolyte balance, managing peripheral circulation cardiovascular function, and regulating body fluid content. Renin is a kidney-produced enzyme that catalyzes the hydrolysis of angiotensinogen (AGT), a precursor synthesized in the liver, resulting in release of limited amounts of angiotensin I (Ang I) [31]. ACE (Angiotensin-converting enzyme) is an additional enzyme that facilitates the cleavage of Ang I into the active octapeptide angiotensin II (Ang II). This peptide is a crucial component of the RAS with a significant role in different physiological activities. The persistent activation of RAS and elevation of Ang II levels can potentially affect AT1R (angiotensin II type 1 receptor), hence initiating inflammation, vasoconstriction, fibrosis, and augmented renal salt reabsorption [32].

When Ang II is administered directly into the brain, it produces a significant increase in blood pressure and, in the presence of adequate water supply, stimulates a response characterized by heightened consumption behaviors. The potential effectiveness of Ang II in reducing hypertrophy may be compromised by several factors, including its intricate three-dimensional structure, the balance of its hydrophilic and hydrophobic characteristics, and overall stability [33]. These considerations contribute to the limited bioavailability of Ang-II [34], rendering it highly susceptible to degradation, with a notably short half-life. This degradation occurs due to the action of enzymes and proteases, which may either attack the compound at injection site or during its journey to the target area where it is intended to exert pharmacological effects. It has been suggested that the influence of Ang II on the central nervous system is primarily localized to the brain itself, largely due to the protective barrier created by the BBB [35].

1.5 Ready to Infuse (RTI) Drug Delivery Systems

Numerous compounds, including peptides and anticancer agents, exhibit instability in aqueous and diluted form. Thus, these compounds are available in lyophilized and concentrated injectable form accordingly. Before being administered, these formulations must be diluted in

appropriate diluents as lactate ringer's solution, dextrose, sodium chloride, or dextrose plus sodium chloride injection. The dilution of injection involves aseptic manipulation, which incorporate additional stage in delivery of medicine to patient. This is very critical when drug is delivered in emergency situation. In addition, physicochemical stability of such diluted formulation is limited up to 24 h at room temperature. The advantages of RTI over concentrated small volume injection is compiled in Table1.1.

Table 1.1: Comparison of RTI dosage form and small volume injections

Sr. No	Parameters	Small volume injection	RTI dosage form
1	Need to dilute	Yes	No
2	Able to control the dose	No	Yes
3	Able to control delivery speed	No	Yes
4	Assurance of availability of drug in the system for longer period	No	Yes
5	Consistent and complete absorption of drug	May be	Yes
6	Stability of drug molecule	Yes	No
7	Opportunity to administer multiple drugs	No	Yes
8	Chances of contamination	Yes	No
9	Dose error	May be	No
10	Manipulation	Yes	No
11	Difficulty in mixing of viscous injection	Yes, some times	No, already mixed

1.6 Nanocarriers-Based Delivery Approach

Nanocarriers are specialized materials that typically measure between 1 - 300 nm in diameter. These tiny carriers are widely employed in the field of medicine for the targeted delivery of drugs and diagnostic agents (Figure 1.2). Their minuscule size allows them to navigate complex biological environments with ease, while their high solubility enhances their ability to transport medications effectively. Additionally, nanocarriers are known for their remarkable selectivity, enabling them to deliver therapeutic agents precisely to the intended cells or tissues. This precision minimizes side effects and maximizes treatment efficacy. Furthermore, these carriers are designed to release drugs at controlled rates, ensuring that the therapeutic compounds are available to the body exactly when and where they are needed.

Nanocarriers exhibit a remarkable phenomenon known as enhanced permeability and retention effect (EPR), which allows them to selectively accumulate in tumor tissues characterized by abnormal and leaky blood vessels [36]. Currently, researchers are investigating a diverse array of nanocarrier systems for the delivery of biologically active compounds and diagnostic agents. These systems include lipid-based carriers that utilize lipid membranes, polymer-based carriers designed for stability and controlled release, lipid-polymer hybrid systems that combine the advantages of both, as well as advanced options like metallic nanoparticles, quantum dots, and carbon nanotubes [37,38]. The design of these sophisticated drug carriers aims to ensure the precise transport of macromolecules to specific target sites within the body while minimizing off-target effects and mitigating both systemic and localized toxicities associated with conventional therapies [37].

1.6.1 Nanocarriers mediated delivery of peptides to brain

Recent groundbreaking advancements in the realm of nanotechnology have opened up a wealth of innovative possibilities for the precise and effective delivery of therapeutic drugs across the of BBB. This significant leap in technology holds tremendous promise for addressing a variety of brain disorders, which have long represented daunting challenges in the field of medical science.

A diverse array of synthetic nanoparticles has emerged as formidable non-invasive carriers for medications aimed at treating brain diseases. Notable examples include nanovesicles, dendrimers, carbon quantum dots, micelles, and polymeric nanoparticles (Figure 1.2) [39]. When assessing attributes such as biocompatibility, safety, and production complexity, natural nanovesicles demonstrate considerable advantages over their synthetic counterparts. These vesicles are designed with aqueous cores surrounded by a lipid or polymer bilayer, effectively encasing medicinal chemicals or active ingredients within their structure. The various forms of these vesicles such as liposomes, exosomes, niosomes, and polymersomes are collectively referred to as vesicular drug delivery systems (VDDSs). These systems play a crucial role in bridging the gap between the hydrophilic and hydrophobic nature of different compounds within a single cohesive delivery platform.

Remarkably, these nanovesicles possess the versatility to encapsulate a wide range of biomolecules, encompassing proteins, lipids, and various types of nucleic acids [40]. Noteworthy example of this technology is hepatocyte-directed vesicles (HDVs), which are specialized liposomes specifically engineered for the targeted encapsulation of insulin. The success of these HDVs is underscored by their commendable completion of Phase III clinical trials, marking a pivotal milestone toward their application in real-world medical treatments. To date, a range of medications employing non-oral vesicle delivery systems have confidently made their way into the commercial market, showcasing their practical viability. Another exciting development is the emergence of salbutamol sulfate-loaded niosomes, which have successfully navigated clinical trials, highlighting their potential as an innovative strategy for delivering medications directly to the lungs. The encapsulation of drugs within vesicles can lead to substantial modifications in critical factors such as their distribution, targeting specificity, bioavailability, and plasma half-life. This transformation underscores the potential of nanovesicles as robust biological drug-delivery systems capable of reliably transporting their therapeutic cargo into targeted cells, thereby enhancing treatment efficacy [40]. To elevate pharmaceutical effectiveness and retrieval, there is a pressing need for cutting-edge therapeutic strategies tailored specifically for patients grappling with neurological disorders. Currently, numerous instances of unmet therapeutic needs have been identified among individuals suffering from various neurological conditions. Patients frequently experience a constellation of negative symptoms, cognitive deficits, and resistance to treatments, compounded by concerns surrounding the safety and tolerability of presently available therapies. These challenges often result in reduced adherence to treatment plans and contribute to stigmatizing behaviors [41]. Furthermore, for any medication delivered systemically to exert its intended therapeutic effects within the central nervous system, it must effectively traverse the endothelium capillary of the BBB.

Progressions in physiological-based methodologies are being harnessed to facilitate the targeted delivery of bioactive substances across the BBB to specific locations within CNS. These innovative techniques exploit intrinsic mechanisms that enable the carrier-mediated delivery of drugs or nutrients, as well as receptor-mediated delivery of peptides [42-44]. Ultimately, the success of neuroprotective treatments will hinge on the bioactive compounds'

ability to effectively interact with, and reach, the designated targets situated within the complex landscape of the central nervous system.

1.7 Conjugation of Peptide Molecules to Enhance the Pharmacokinetics and Pharmacodynamic Attributes

Altering peptides through various modification techniques leads to noteworthy improvements in their stability, selectivity, and overall biological activity, which are crucial for their effectiveness as therapeutic agents. Among the most prominent examples of these successful chemical modifications in clinical use today are semaglutide, liraglutide, and selepressin, each demonstrating significant enhancements in their pharmacological profiles. However, it is important to note that certain chemical modifications can fall short in achieving a simultaneous boost in selectivity, activity, and stability within the context of the proteolytic processes involved. In contrast, peptides that undergo modifications with D-amino acids often fail to display the desired levels of biological activity, limiting their therapeutic potential [45-47]. Overall, these peptide modifications serve to enhance the drug-like characteristics of peptides, effectively improving their biological performance and extending their stability in plasma. [48].

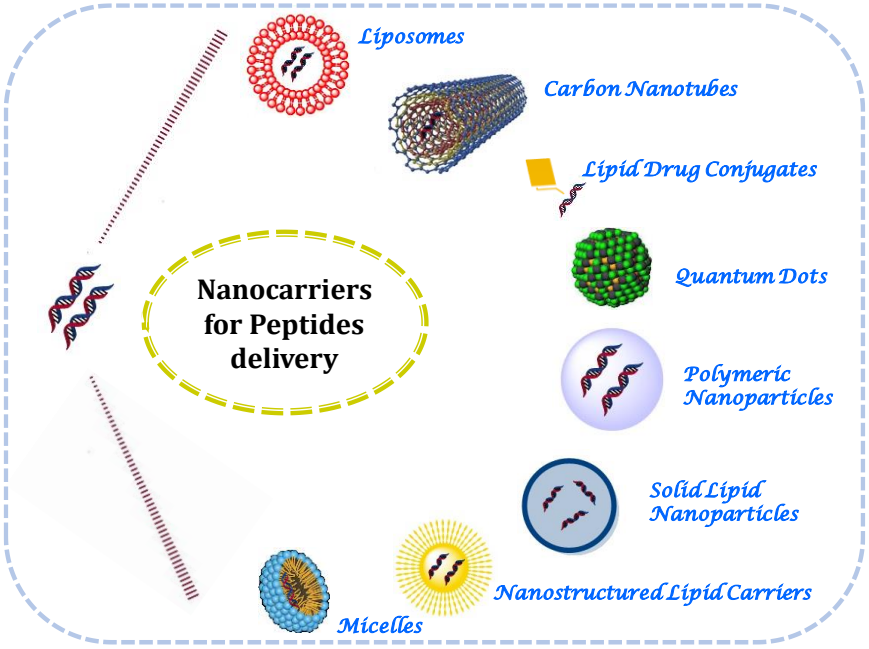

Figure 1.2: Various nanocarriers for delivery of peptides

1.8 Aims & Objectives

1.8.1 Aims

Considering the challenges linked to peptide therapeutics, including low bioavailability, short half-life, limited ability to penetrate the brain through oral or intravenous routes, and stability concerns like sensitivity to oxygen and light. This study focused on designing, developing, and optimizing effective delivery systems for oxytocin, vasopressin, and angiotensin-II to address these issues appropriately.

1.8.2 Ready to infuse (RTI) formulation

The aforementioned limitations create a need for ready to administer formulation of peptide molecules which can be directly administered to patient. Moreover, parenteral drug manufacturing of oxygen/photosensitive drug in plastic infusion bag becomes critical due to the semipermeable nature of infusion bag, as it allows oxygen to penetrate semipermeable layer of infusion bags. However, few infusion bags are available which are made up of materials that restrict oxygen to permeate inside the bag. With insight of reported problems during dilution and associated stability issues, proposal to design, develop and optimize suitable RTI delivery systems of oxytocin, vasopressin and angiotensin-II. RTI approach will provide a composition and process for developing injection of sensitive and potent molecules. RTI dosage forms also provide better compliance and safety to the patient. The maximum safety is prominent factor in RTI due to its uniqueness in zero error during dose administration and minimum cross contamination.

The proposed "Ready to Infuse" dosage form will be novel and unique in terms of strategy for administration, compositions and dosage form, which can be patentable and can be commercialized.

1.8.3 Nano-formulations for nose to brain delivery

In order to improve the stability, shelf life and bioavailability of oxytocin and vasopressin, it was proposed to design and develop their nano-delivery systems for management of AD. It was postulated that the designed dosage form will provide a composition and process for

formulating injection and delivery *via* intranasal route of these sensitive molecules in order to provide better compliance and safety for the patient.

The proposed hypothesis is unique and novel in terms of dosage form, strategy for administration, compositions of delivery systems, which enhances patentability as well as commercialization aspects and can be commercialized.

1.8.4 Chemical modification to improve half-life and bioavailability of oxytocin

Oxytocin has the very short half-life in blood circulation (4- 10 min). Therefore, it was selected as a model molecule for further modifications. On the path of addressing bioavailability and short half-life of oxytocin, the aim of present study was to improve retention time of oxytocin in body along with enhancement in permeation of oxytocin across BBB through conjugation. The 2-(2-(2-Aminoethoxy) ethoxy) acetic acid (AEEA) conjugation with one of the amino acids of oxytocin may improve the pharmacokinetic properties of oxytocin.

1.9 Objectives

The objectives of present work have been divided in three major sections as mentioned below:

1.9.1 Ready to infuse (RTI) drug delivery system

1) To design, develop and characterize oxytocin based RTI injection
2) To design, develop and characterize angiotensin-II based RTI injection
3) To design, develop and characterize vasopressin based RTI injection

1.9.2 Nano-formulations for nose to brain delivery

1) To design and develop oxytocin-loaded nanovesicles for brain delivery
2) To design and develop vasopressin-loaded nanovesicles for brain delivery

1.9.3 Oxytocin-conjugates for nose to brain delivery

1) To execute conjugation of 2-(2-(2-Aminoethoxy) ethoxy) acetic acid (AEEA) with oxytocin.
2) To characterize and evaluate the prepared conjugates.

1.10 Plan of Work

1.10.1 Development and Characterization of Ready to Infuse Formulations

1. Pre-formulation studies and analytical method development
2. Optimization and development of RTI formulations of oxytocin, vasopressin and angiotensin-II
3. Physicochemical characterization of developed formulations
4. *In vitro* cell line studies
5. Stability studies

1.10.2 Development and Characterization of Nano-formulations for Nose to Brain Delivery

1. Nano formulation development for oxytocin and vasopressin
2. Physicochemical characterisation of developed formulations
3. Cell line studies (*In vitro* cellular uptake and cytotoxicity studies)
4. *In vivo* pharmacodynamics studies
5. Stability studies

1.10.3 Synthesis and Characterization of Oxytocin Conjugate for Nose to Brain Delivery

1. Synthesis of oxytocin conjugate with 2-(2-(2-Aminoethoxy) ethoxy) acetic acid
2. Physicochemical characterisation of conjugate
3. Cell line studies (*In vitro* cellular uptake and cytotoxicity studies)
4. *In vivo* pharmacodynamics studies

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