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Optimization and Development of Supramolecular Cage for Augmented Oral Insulin Delivery

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ABSTRACT

Diabetes mellitus is a chronic metabolic disorder marked by elevated blood glucose due to either insufficient insulin production or insulin resistance. Insulin therapy, especially for Type 1 diabetes, is essential but typically involves painful subcutaneous injections, leading to poor patient compliance and discomfort. To address this, **oral insulin delivery** is being actively explored due to its non-invasive nature, enhanced patient adaptability, and ability to mimic natural insulin pathways by reaching the liver via the portal vein.

However, oral delivery of insulin faces major challenges due to the complex gastrointestinal (GI) environment. Insulin is a peptide hormone with high hydrophilicity, large molecular weight, and low stability, making it vulnerable to **enzymatic degradation and poor intestinal absorption**. These barriers significantly reduce its oral bioavailability.

Despite considerable research and ongoing clinical trials, no commercial oral insulin product has yet successfully addressed all these limitations. The lack of a viable formulation is due to the structural and physiological complexity of the GI tract.

To overcome these obstacles, several innovative formulation strategies have been developed using **supramolecular cucurbit [6]uril cages to encapsulate insulin**, providing a protective barrier against gastric acid degradation and enhancing its stability for effective oral delivery.

KEY WORDS: Oral insulin delivery, cucurbituril[6], tween 80, phenylalanine

INTRODUCTION

Diabetes mellitus is a widespread metabolic disorder and a major global health concern, contributing to millions of deaths each year. It is primarily managed using parenteral insulin injections, which, although effective, often lead to local tissue complications and do not mimic the natural physiological route of insulin action [1]. As a result, oral insulin delivery has emerged as a promising alternative due to its ability to simulate endogenous insulin transport through the hepatic portal circulation, thereby enhancing patient compliance and comfort [2].

However, the development of oral insulin formulations faces significant hurdles, including enzymatic

degradation in the gastrointestinal (GI) tract, poor permeability across the intestinal epithelium, and structural instability of the insulin molecule [3]. To address these challenges, innovative strategies have been explored. These include the use of absorption enhancers, protease inhibitors, submicron delivery carriers, enteric coatings, mucoadhesive systems, structural modifications, and techniques such as iontophoresis to improve bioavailability and targeted delivery.[4]

Insulin itself is a peptide hormone consisting of two chains—A and B—linked by disulfide bonds, playing a crucial role in glucose homeostasis and overall metabolic regulation. Since its discovery in 1921, insulin has remained a foundational therapy for diabetes, especially in patients with impaired natural insulin production [5]. The disease presents in several forms, including Type I, Type II, gestational, and monogenic diabetes, each with distinct causes ranging from autoimmune destruction of β-cells to genetic and lifestyle influences.

Advances in biotechnology have enabled mass production of recombinant human insulin using microbial expression systems, while the development of insulin analogs has further improved therapeutic outcomes by providing rapid onset of action, better glycemic control, and reduced risk of hypoglycaemia[6].

Barriers of oral insulin delivery

The gastrointestinal (GI) tract is lined by a single layer of tightly connected columnar epithelial cells. These cells are supported by the lamina propria and the muscularis mucosa. Tight junctions, also known as zonula occludens, link adjacent epithelial cells and significantly restrict the paracellular passage of molecules, creating a selective barrier to absorption [7].

To increase the absorptive surface area, the intestinal epithelium forms folds called villi, and the apical surfaces of the epithelial cells are covered in microvilli—together forming the brush border. These structural adaptations amplify the surface area of the intestine by roughly two orders of magnitude. However, they also complicate the absorption of protein-based drugs, as the brush border contains digestive enzymes that can degrade proteins before absorption.[8]

Further contributing to this barrier is the glycocalyx—a coating of sulfated mucopolysaccharides—and an overlying mucus layer composed of glycoproteins, enzymes, water, and electrolytes. These additional layers add to the physical obstruction faced by orally administered proteins.[9]

Beyond these physical barriers, a significant enzymatic barrier exists within the GI tract. Protein digestion begins in the stomach with enzymes like pepsin and continues in the small intestine with pancreatic enzymes such as trypsin, chymotrypsin, and carboxypeptidases[10]. However, only about 20% of enzymatic degradation is carried out by these luminal enzymes. Most of the protein breakdown occurs at the brush-border membrane via peptidases or inside the intestinal epithelial cells (enterocytes). For instance, insulin is further broken down by a specific cytosolic enzyme known as insulin-degrading enzyme.[11]

Efforts to overcome these enzymatic challenges have proven difficult. Maintaining protein integrity is particularly complex due to their sensitivity to environmental conditions[12]. Factors such as temperature, pH, solvent interactions, solute presence, and the crystalline state can all destabilize a protein's structure. Proteins rely on precise primary (amino acid sequence), secondary (e.g., alpha-helices), tertiary (3D folding), and quaternary (subunit arrangement) structures to remain biologically active. Even slight environmental changes can disrupt these structures, leading to a loss of function.[13]

This sensitivity poses a significant challenge in developing polymer-based drug delivery systems. While

encapsulation techniques aim to protect proteins during transport and release, many common fabrication methods can inadvertently
damage the protein's structure, rendering it inactive. Therefore, in addition to analysing release kinetics, ensuring the structural stability of the protein is critical during formulation development.[14]
To improve oral protein delivery such as that of insulin various strategies have been investigated. These often aim to address either the epithelial barrier or the enzymatic degradation. A widely used approach involves the use of penetration enhancers, including surfactants, fatty acids, and bile salts. These agents can increase epithelial permeability and facilitate transport by loosening tight junctions and thinning the mucus layer.[15]

EXPERIMENTAL SETUP

Methodology Preparation of solutions

In the present study, recombinant **Human Insulin (rDNA origin)** was utilized as the model therapeutic protein to evaluate the formulation strategy. The insulin used was procured under batch number **RHI24105**, ensuring traceability and consistency throughout the experimental process. This specific batch was selected based on its documented purity and stability profile, which are critical parameters for assessing both the integrity of the protein and the reliability of the drug delivery system under investigation**.[Brought from M.J. Pharma Private Limited, Pune]**
Cucurbit [6]uril (CB[6]), a member of the cucurbituril family, is a macrocyclic host molecule known for its rigid, symmetrical structure and exceptional ability to form stable host–guest complexes. Due to its hydrophobic cavity and polar carbonyl-laced portals, CB [6] exhibits remarkable affinity toward various organic and inorganic guests, particularly ammonium and alkylammonium ions. Its unique physicochemical characteristics make it a powerful tool in supramolecular chemistry, enabling applications in drug delivery, molecular recognition, catalysis, and materials science[16]. In this work, CB[6] was procured from **Otto Chemie Pvt. Ltd. (India**), was used as **supramolecular cage to protect insulin** from stomach degradation, ensuring high purity suitable for reproducible experimental outcomes and further structural investigations.
The compound's inclusion capabilities and complexation behaviour continue to drive innovation in fields ranging from nanotechnology to biomedicine**.**
To the best of our knowledge, **cucurbit [6]uril (CB[6]) has not yet been explored for this particular application**. In this study, we have chosen to investigate its potential due to its unique host–guest chemistry and promising physicochemical properties.

Adverse effects of Cucurbit[6]uril in oral insulin

Despite the robust host–guest chemistry of cucurbit[6]uril (CB[6]), its application in oral insulin delivery remains limited. This is primarily attributed to its relatively small cavity size (~5.8 Å), which restricts its ability to encapsulate large biomolecules such as insulin (~5.8 kDa) effectively.[17]

Strategies implemented to overcome this barrier

To address the inherent limitations of CB[6] in encapsulating macromolecular therapeutics such as insulin, co-guest molecules like Tween 80 and phenylalanine have been employed. These co-guests modulate the host cavity environment and enhance the complexation efficiency of CB[6], thereby improving solubility, stabilization, and potential translocation across gastrointestinal barriers[18]. In macrocyclic host systems such as cucurbiturils, cyclodextrins, and other supramolecular cages, co-guest molecules play a critical role by interacting with the host–guest complex to further influence the

physicochemical properties of the encapsulated drug. Specifically in insulin delivery, co-guests can enhance or modulate encapsulation, stability, solubility, and release profiles. Commonly used co-guests include surfactants, amino acids, polyols, and sugars. Among these, surfactants are particularly effective in enhancing both the permeability and solubility of insulin, thereby improving its oral bioavailability."[19]

TWEEN 80

Polysorbates, commonly known as Tween, are nonionic surfactants widely utilized in pharmaceutical formulations, including parenteral drugs and vaccines. These surfactants primarily consist of fatty acid esters of polyethoxylated sorbitan, with Tween 20® and Tween 80® (polysorbate 20 and polysorbate 80, respectively) being among the most commonly used variants.

The composition of Tween formulations is complex, comprising ethoxylated oligomers with various fatty acid chains, ethoxylated sugars, and polyethylene glycol (PEG) oligomers[20].

Tween 80 improves the solubility and dispersion of CB[6]. CB[6] is poorly water-soluble. Tween 80 reduces the hydrophobic interaction and also prevents CB[6] aggregation. This also improves the solubility and stability of CB[6]- insulin complex. Tween 80 prevents the degradation and aggregation of insulin from stomach acid.[21]

Tween 80 forms a protective micelle around the CB[6]-insulin complex to stabilize insulin, and prevent degradation. Tween 80 increases permeability across intestinal membranes by modifying the lipid structure of cell membranes, allowing better insulin absorption. Tween 80 can modify the release profile of CB[6]-insulin complexes, allowing for sustained insulin release in the intestine. The advantages of Tween 80 in CB[6] oral insulin delivery are increased solubility, preventing aggregation, disruption of intestinal lipid barriers, and modified diffusion rate.[22] [23] [24]

Phenylalanine:

Structure

Phenylalanine (abbreviated as Phe or F) is an essential α-amino acid with the molecular formula $C_9H_{11}NO_2$. Structurally, it can be considered as an alanine molecule where a benzyl group replaces the methyl group or, alternatively, as an alanine derivative with a phenyl group in place of a terminal hydrogen. Due to the nonpolar and hydrophobic nature of its benzyl side chain, phenylalanine is classified as a neutral and nonpolar amino acid. [25]

The biologically relevant L-isomer of phenylalanine is incorporated into proteins as directed by the genetic code. This amino acid serves as a precursor to tyrosine and plays a crucial role in the biosynthesis of key neurotransmitters, including dopamine, norepinephrine (noradrenaline), and epinephrine (adrenaline). Additionally, phenylalanine contributes to melanin production and is encoded by the messenger RNA codons UUU and UUC.[26]

Phenylalanine is an amino acid used as a co-guest molecule for enhancing the encapsulation, stability, and absorption of insulin. Phenylalanine has an

aromatic benzyl group that interacts with CB6 via π-π stacking. To stabilize insulin encapsulation, phenylalanine forms hydrogen bonds with the carbonyl portals of CB6. By acting as a bridge between CB6 and insulin phenylalanine can improve complexation efficiency[27]. During formulation and storage, it helps to maintain the structural integrity of insulin. The mechanism of

interaction between phenylalanine and CB6 occurs through π-π Stacking Interactions, Hydrogen Bonding with CB6 Carbonyl Portals, Competitive & Cooperative Binding with Insulin.[28]

Phenylalanine can either compete or cooperate with insulin for CB6 binding, depending on its concentration. Phenylalanine helps insulin bind to CB6 by bridging interactions at low concentrations. It may also compete with insulin for CB6 occupancy, at high concentrations, potentially reducing insulin encapsulation.[29]

EXPERIMENTAL PROTOCOL

An acetate buffer (pH 4.5) was prepared as the solvent system for all subsequent steps. Insulin (10 mg) was dissolved in 5 mL of the prepared buffer and stored in a separate flask. In parallel, CB[6] (10 mg) was dissolved in 5 mL of the same buffer in a separate vessel. Both the insulin and CB[6] solutions were subjected to magnetic stirring to ensure complete dissolution. The CB[6] solution was then sonicated, during which co-guest molecules—Tween 80 (10 mL) and phenylalanine (10 mg)—were introduced to facilitate host–guest complexation. Following co-guest incorporation, the CB[6] solution was combined with the insulin solution under gentle stirring. The resulting formulation was then stored at low temperature using a refrigerated cabinet to preserve its structural integrity and stability."

RESULTS & DISCUSSION

Comparative Analysis of DLS Results: Insulin vs. Insulin–CB6 Complex

Dynamic Light Scattering (DLS) measurements were conducted to assess the hydrodynamic size distribution and polydispersity of Insulin (Sample 1) and the Insulin–CB6 complex (Sample 2) at 25°C in distilled water (refractive index: 1.3328; viscosity: 0.8878 cP).

Key DLS Parameters:

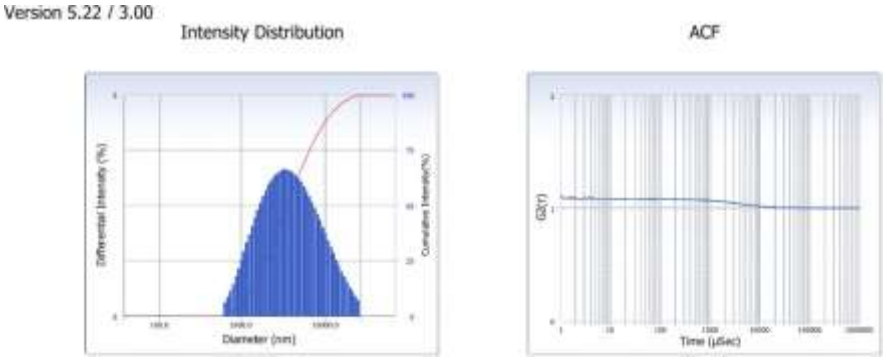
Parameter	Sample 1: Insulin	Sample 2: Insulin–CB6 Complex
Z-Average Diameter (nm)	2,901.3	4,549.5
Polydispersity Index (PDI)	0.458	0.969
Diffusion Coefficient (cm²/s)	1.695×10^{-9}	1.081×10^{-9}
Scattering Intensity (cps)	29,333	33,939
Residual Error	2.524×10^{-3}	3.155×10^{-3}
Intensity Peak (nm)	5,452.0	13,795.3
Standard Deviation (nm)	4,789.6	16,089.6
D10 (nm)	1,279.9	1,633.2
D50 (Median, nm)	3,671.6	7,271.7
D90 (nm)	11,577.3	33,684.5

- **Hydrodynamic Size & Aggregation**: The Insulin–CB6 complex (Sample 2) exhibits a larger Z-average diameter (4,549.5 nm) compared to Insulin alone (2,901.3 nm), indicating the formation of larger aggregates upon complexation.
- **Polydispersity**: Sample 2 has a higher PDI (0.969) than Sample 1 (0.458), suggesting a broader size distribution and greater heterogeneity in particle sizes, which is characteristic of a highly polydisperse system.
- **Diffusion Coefficient**: The lower diffusion coefficient in Sample 2 (1.081×10^{-9} cm²/s) relative to Sample 1 (1.695×10^{-9} cm²/s) corroborates the presence of larger particles in the Insulin–CB6

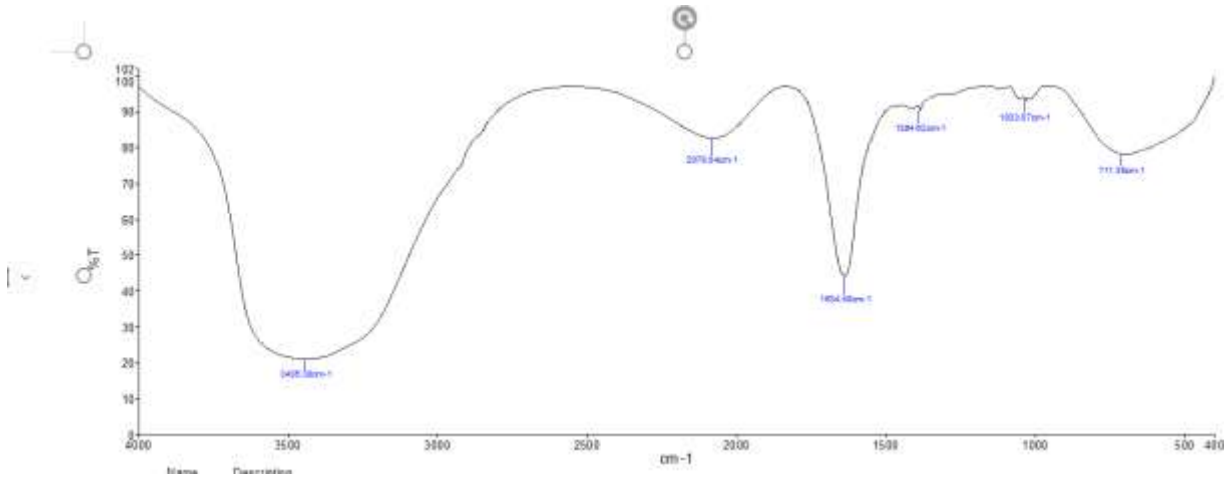
complex.

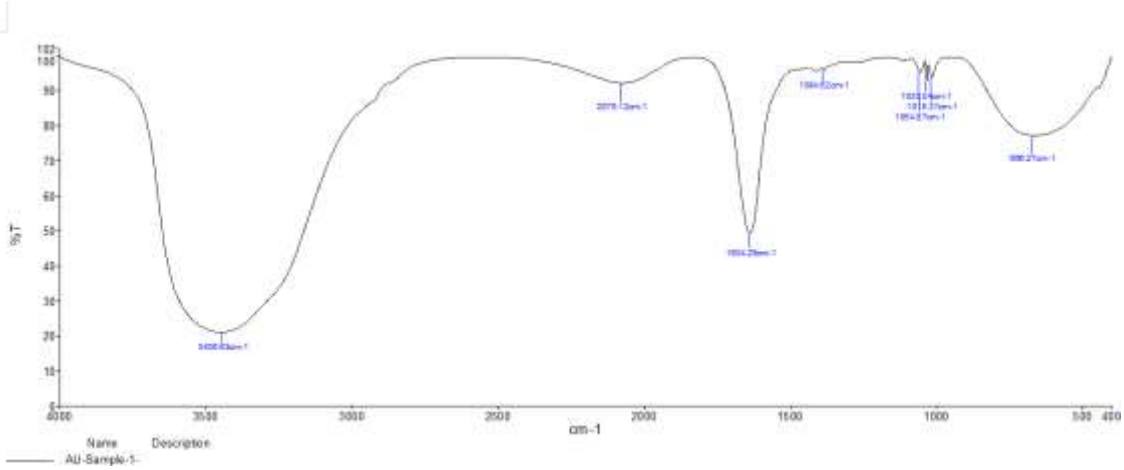
- **Size Distribution Percentiles**: The D50 value for Sample 2 (7,271.7 nm) is nearly double that of Sample 1 (3,671.6 nm), and the D90 value is approximately three times higher, indicating a significant shift towards larger particle sizes in the complexed form.
- **Scattering Intensity & Model Fit**: Both samples exhibit acceptable residual errors (Sample 1: 2.524 × 10^{-3}; Sample 2: 3.155 × 10^{-3}), indicating reliable model fits. The higher scattering intensity in Sample 2 (33,939 cps) compared to Sample 1 (29,333 cps) further supports the presence of larger or more numerous scattering centres in the complex.

The DLS analysis reveals that the Insulin–CB6 complex forms larger and more heterogeneous aggregates compared to Insulin alone. The increased hydrodynamic size, higher polydispersity index, and altered diffusion coefficient in Sample 2 suggest that CB6 promotes significant self-assembly or aggregation of Insulin molecules, which may have implications for the complex's stability and functional behaviour in pharmaceutical applications.[30] [31].



Comparative FTIR Analysis of Insulin and Insulin–CB6 Complex





The comparative FTIR spectra of insulin (Sample 1) and the insulin–CB6 complex (Sample 2) reveal key insights into the nature of their interaction and the protective effect of CB6 on insulin stability. Both samples exhibit nearly identical absorption bands characteristic of insulin's molecular structure, particularly the amide I band around 1634 cm^{-1}, which is indicative of the
protein's secondary structure. The minimal shift between the two samples (1634.29 cm^{-1} vs. 1634.40 cm^{-1}) suggests that insulin maintains its native conformation in the presence of CB6, with no significant alteration in its peptide backbone.[32]
Similarly, broad O–H/N–H stretching bands (~3436 cm^{-1}) and C–H bending vibrations (~1384 cm^{-1}) show negligible changes, indicating that the functional groups of insulin are not chemically modified upon mixing with CB6. Minor shifts observed in lower frequency regions, such as the out-of-plane bending vibration moving from 666.27 cm^{-1} to 711.35 cm^{-1}, likely reflect weak physical interactions or steric effects rather than strong chemical bonding.[33]
Overall, the FTIR data suggest that **CB6 does not form a strong covalent** or hydrogen bond complex with insulin but i**nstead protects insulin through physical encapsulation or steric shielding**.[34] This non-disruptive interaction effectively preserves insulin's molecular integrity, implying that CB6 can stabilize insulin against degradation without altering its native structure. Such a mechanism holds promise for enhancing insulin stability in pharmaceutical formulations while maintaining its biological activity.[35]

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