Journal of Molecular Science

Journal of Molecular Science
Volume 35 Issue 3, Year of Publication 2025, Page 775-781
DoI-10.004687/1000-9035.2025.104

Journal of Molecular Science
www.jmolecularsci.com ISSN:1000-9035

Formulation and Evaluation of Novel Anticoagulant Apixaban Loaded Cubosomes by using experimental design technique

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Article Information
Received: 11-09-2025
Revised: 08-10-2025
Accepted: 25-10-2025
Published: 14-11-2025

Keywords
Apixaban, Cubosomes, Particle Size, Drug Entrapment Efficiency

ABSTRACT

This study investigates the formulation, characterization, and evaluation of Apixaban (APX)-loaded cubosomes as a novel nanocarrier system designed to enhance the drug's therapeutic profile. Apixaban, a highly selective factor Xa inhibitor, is a BCS Class III drug with low permeability and limited aqueous solubility (0.04 mg/mL), contributing to its moderate oral bioavailability (~50%). Cubosomes, distinct sub-micron liquid crystalline particles composed of amphiphilic lipids like Glyceryl Monooleate (GMO) and stabilized by Poloxamer 407, were employed to improve APX solubilization and achieve controlled release. Formulations were prepared using a blend of APX, GMO, and Poloxamer 407 via both top-down and bottom-up approaches. Five formulations (F1−F5) were evaluated, with F4 demonstrating the optimal characteristics: a desirable particle size (251.8 nm), a highly negative zeta potential (−79.0 mV) indicating good colloidal stability, a low polydispersity index (PDI) (2.15%), and an exceptional Drug Entrapment Efficiency (DEE) of 96%. The in-vitro diffusion study revealed that formulation F4 achieved the highest cumulative drug release (84±0.12%) after 6 hours, confirming its superior controlled release performance. Furthermore, a long-term stability study of the optimized F4 batch at room temperature (27∘C) showed minimal changes over three months in particle size (251.8 nm to 252.8 nm) and DEE (96% to 95.4%), confirming its physical robustness. These results validate that APX-loaded cubosomes, particularly the optimized F4 formulation, offer a promising, stable, and highly effective nanoplatform for the enhanced oral delivery of Apixaban.

INTRODUCTION:

Apixaban (APX) is a direct, highly selective, and reversible inhibitor of the activated coagulation factor X (FXa), whose effect does not depend on antithrombin for antithrombotic action. The drug acts by inhibiting the FXa (free and bound) to the clot and also the activity of prothrombinase, thereby preventing the generation of thrombin and thrombus formation. Apixaban is indicated for the prevention of venous thromboembolism. Apixaban is currently sold under the trade name of Eliquis (Bristol-Myers and Pfizer, USA) as coated tablets at dosages of 2.5 and 5 mg. APX has an acidic pKa of 13.12 and a basic of -1.60, therefore it does not ionize at physiological pH and remains in its neutral form. Apixaban is crystalline, non-hygroscopic, white to a yellowish white substance

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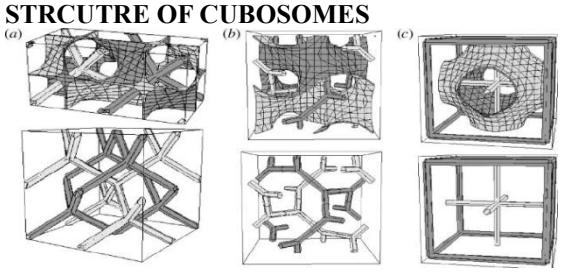
that is immiscible in water but soluble in DMSO (Di -methyl Sulfoxide). The chemical name of apixaban is 1-(4-methoxyphenyl)-7-oxo-6-[4-(2-oxopiperidin-1-yl) phenyl]-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c] pyridine-3-carboxamide. **C**onferring to the Biopharmaceutical Classification System, APX is a class III molecule with high solubility and low permeability. It has an oral bioavailability around 50% and aqueous solubility of about 0.04 mg/mL in the physiological pH range pH 1.2–6.8. Apixaban is one of the novel anticoagulants to emerge as alternatives to long-standing standards of care that include low-molecular-weight heparin and warfarin. The new oral anticoagulants target competitive inhibition of single enzymes in the coagulation cascade, leading to anticoagulant effects directly related to their concentration.[1-3]

CUBOSOMES

Amphiphilic lipids possess very low aqueous solubility and often self-assemble into lyotropic liquid crystalline phases in the presence of excess water. Cubosomes are square and rounded particles with internal cubic lattices visible. Cubosomes are distinct, sub-micron, nano-structured particles of bicontinuous cubic liquid crystalline phase. They are formulated by certain amphiphilic lipids as glycerol monooleate (GMO) and phytantriol (PHYT), which have the ability to self-assemble in water to form cubosomes. They encompass a structure similar to honeycomb (cavernous) structures with a size range of 100-500 nm. Cubic phase consists of a curved bi-continuous lipid bilayer extending in three dimensions and separating two congruent networks of water channels so it can enclose hydrophilic, amphiphilic, and hydrophobic substances. Naturally, three forms of cubic phase have been used as drug delivery systems: cubic phase gel, cubic phase precursor, and cubosomes. Cubosomes can be considered as a novel lipid-based Nano systems similar to well-known vesicular systems such as liposomes and niosomes. They can represent a novel drug delivery system which could be loaded with hydrophilic, lipophilic and amphiphilic drug molecules. They can potentially increase the absorption of the drug and extend its release owing to their lipid bilayer, which is structurally similar to the lipid bilayer of biological membranes.

STRCUTRE OF CUBOSOMES



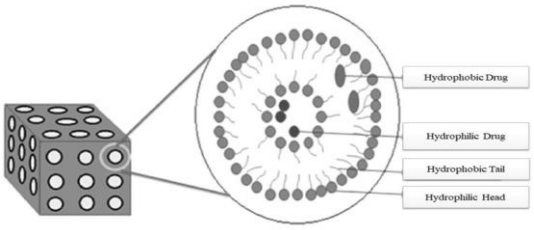


Figure 1: Structure of Cubosome

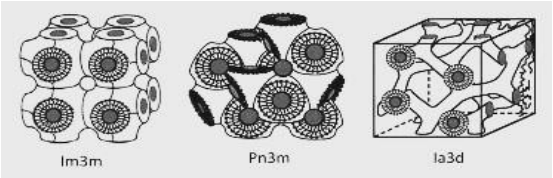


Figure 2: Underlying minimal surfaces and skeletal graphs (centre of water channels) for the inverse bicontinuous cubic phases. (a) Pn 3 m (Diamond), (b) Ia 3 d (Gyroid) and (c) Im 3 m (Primitive).

THEORIES OF CUBOSOMES[4]

Cubosomes are fascinating nanostructures used in drug delivery systems. They are formed by the self-assembly of amphiphilic lipids in water, creating a bicontinuous cubic phase. Here are some key theories and concepts related to cubosomes:

1. Self-Assembly Mechanism

Cubosomes form through the self-assembly of amphiphilic molecules, such as glyceryl monooleate (GMO) and phytantriol (PHYT), in an aqueous environment. This process is driven by the hydrophobic and hydrophilic interactions of the lipid molecules.

2. Bicontinuous Cubic Phase

The internal structure of cubosomes is a bicontinuous cubic phase, which means it has a three-dimensional, periodic structure with continuous channels of both water and lipid. This unique structure allows cubosomes to encapsulate and deliver a variety of drug molecules, including hydrophilic, hydrophobic, and amphiphilic compounds.

3. Drug Delivery Applications

Cubosomes are highly versatile and can be used for various drug delivery applications, such as:

- **Oral Delivery**: Enhancing the bioavailability of poorly water-soluble drugs.
- **Ocular Delivery**: Providing sustained release of drugs for eye conditions.
- **Transdermal Delivery**: Delivering drugs through the skin.
- **Cancer Therapy**: Targeting tumor cells with minimal side effects.

4. Advantages of Cubosomes

- **High Drug Loading Capacity**: Due to

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their unique structure, cubosomes can encapsulate a large amount of drug.

- **Biocompatibility**: Made from biodegradable lipids, cubosomes are generally safe for use in the body.
- **Controlled Release**: They can provide sustained and controlled release of drugs, improving therapeutic outcomes.

MATERIALS:

Apixaban drug was given gift sample by CTX LiFESCIENCES PVT. LTD., Gujarat. All other excipients was provided by Mohini Organics Pvt. Ltd, Mumbai.

METHODS USED IN PREPARATION OF CUBOSOMES[5-6]

Cubosomes can be prepared using two main methods: the **Top-Down** and **Bottom-Up** approaches. Here's a detailed explanation of each method along with a flowchart for better understanding:

1. Top-Down Method

This method involves breaking down a bulk cubic phase into smaller cubosome particles. It typically requires high shear forces to disperse the cubic phase into nanoparticles.

Steps:

1. **Preparation of Bulk Cubic Phase**: Mix the lipid (e.g., glyceryl monooleate) with water to form a bulk cubic phase.
2. **High-Shear Homogenization**: Apply high shear forces using a homogenizer to break down the bulk cubic phase into smaller cubosome particles.
3. **Stabilization**: Add a stabilizer like Poloxamer 407 or Poloxamer 188 to prevent aggregation and maintain the stability of the cubosomes.

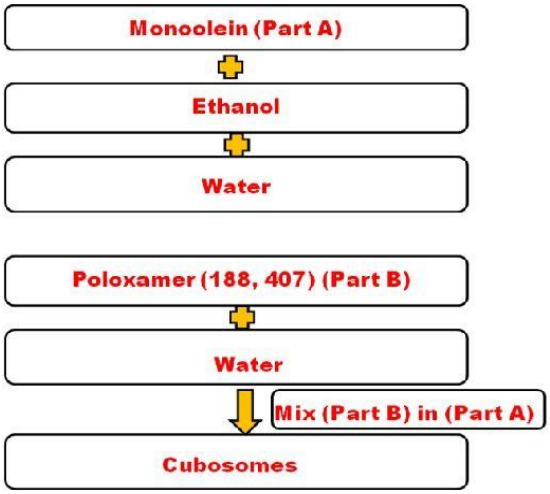

Figure 3: Flow Chart Preparation of Cubosomes Form by Dilution of an isotropic solution

2. Bottom-Up Method

This method involves the self-assembly of lipids into cubosomes from molecular precursors in an aqueous environment.

Steps:

1. **Dissolution of Lipid**: Dissolve the lipid in ethanol or another suitable solvent.
2. **Addition of Aqueous Phase**: Slowly add the aqueous phase containing a stabilizer (e.g., Poloxamer 407) to the lipid solution.
3. **Self-Assembly**: Allow the lipids to self-assemble into cubosomes upon dilution with water.
4. **Stabilization**: Ensure the cubosomes are stabilized with the added stabilizer to Maintain Their Structure.

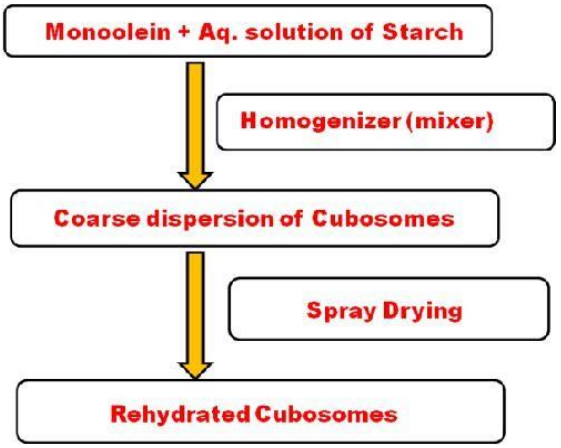

Figure 4: Flow Chart of Preparation of Powdered Cubosomes

Table 1: Formulation Table

Formulation Code	Drug (mg)	GMO (ml)	Poloxamer 407 (mg)	Water (ml)
F1	50	2	1.5	50
F2	50	4	2.5	50
F3	50	6	1.5	50
F4	50	2	2.5	50
F5	50	4	1.0	50
F6	50	6	2.5	50

EVALUATION STUDY:[7-13]

Particle Size Analysis:

- The size distribution of the particles in a sample is described by its particle size.
- Solid materials, Cubosomes, suspensions, emulsions, and even serosols can all be subjected to particle size analysis.
- Particle sizes are measured using a variety of techniques.
- Using a Horiba scientific instrument and the laser diffraction method, the particle size of the prepared cubosomes was determined.

ZETA POTENTIAL

- The more evenly charged particles there are, the stronger the electrostatic force between them, and the more stable the physical

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structure.

- So called zeta potential, which is determined, for example, by the electrophoretic mobility of the particles in an electrical field, is commonly used to quantify the particle charge.
- Colloid titration can also be used to quantify the particle charge in surface charge per surface unit.

POLYDISPERSITY INDEX (PDI):

A sample's heterogeneity is gauged by its size using the polydispersity index (PI). A sample's size distribution or agglomeration or aggregation during isolation or analysis can both result in polydispersity.

Formula:

PDI = Mw / Mn

- Mw is the weight-average molar mass.
- Mn is the number-average molar mass.

DRUG ENTRAPMENT EFFICIENCY (DEE) PROCEDURE

1. 10 ml prepared Cubosomal formulation was taken and it add in a test tube.
2. After that test tube place in centrifuge machine.
3. Formulation was centrifuge at 15000 RPM for 90 min.
4. Then the supernatant was collected and filtered.
5. Then 1 ml filter supernatant was collected and diluted with water up to 10 ml.
6. Finally measured the absorbance of each sample at 230 nm wavelength.

Formula:

DEE = Actual yield x 100
Theoretical yield

DRUG CONTENT:

PROCEDURE

1. Sample Collection.
2. Sample Preparation.
3. Standard Preparation.
4. Instrument Calibration.
5. Analysis.
6. Calculation of Drug Content
7. Result.

FORMULA

EE = Total DC - supernatant DC X100
Total drug conc

ELECTRON MICROSCOPE:

- The intrinsic structure of dispersed cubic vesicles can be identified by electron microscope.
- The synthesis of apixaban cubosomal vesicles was confirmed in this study by the observation.
- The images of cubic nanoparticles with dimensions varying different sizes of nano particles.

IN – VITRO DIFFUSION RELEASE STUDY:

- The dialysis membrane diffusion technique was employed to evaluate the drug release from the Cubosomes.
- This method, 10 mL of the Sample was placed in the donor compartment of a diffusion cell apparatus.
- The donor compartment was securely positioned within a water-jacketed beaker containing 300 mL to maintain sink conditions.
- The entire assembly was maintained at a controlled temperature of 37 ± 1°C for 24 hours.
- The beaker contents were continuously stirred using a magnetic stirrer to ensure uniform mixing.
- At predetermined intervals, 5 mL samples were withdrawn from the receptor compartment and promptly replaced with an equal volume of fresh medium to preserve sink conditions.
- The withdrawn samples were analyzed for drug content using a UV spectrophotometer at a wavelength of 238 nm.
- This precise and controlled setup ensured reliable measurement of the drug release profile from the Cubosomes, reflecting its dissolution kinetics and release characteristics.

STABILITY STUDY OF APIXABAN CUBOSOMES AT ROOM TEMPERATURE

- The stability study is conducted under controlled room temperature conditions, typically at 27°C, in accordance with International Council for Harmonization (ICH) guidelines Q1A (R2) for long-term stability studies.
- Physical stability is monitored to detect changes in particle size or aggregation, as these could compromise the therapeutic effectiveness of the formulation.

RESULTS AND DISCUSSION

Physical Appearance:

- Colour- White to pale yellowish powders
- Odour- No odour.
- Nature- Powder.

Melting Point:

- Apixaban- 237 °C.

Solubility:

- Water (pH) - Poor solubility, cloudy suspension.

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- Methanol - Slightly turbid, low solubility.
- Ethanol – Clear solution, excellent solubility.
- N, N-Dimethyl formamide – Clear solution, excellent solubility.
- Dimet
- hyl sulfoxide – Clear solution, excellent solubility.

Chloroform - Clear solution, excellent solubility.

CALIBRATION CURVE:

Table no: 02:Calibration Curve

Concentration (µg/ml)	Absorbance
0	0
2	0.1503
4	0.3041
6	0.4780
8	0.6293
10	0.8079

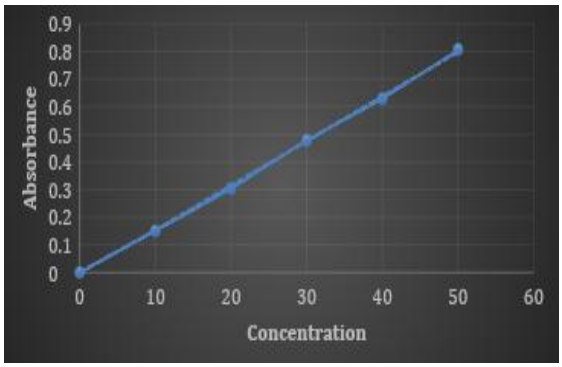

Figure no: 05 – Calibration Curve

➢ **FTIR:**

Figure no: 06 – FTIR

PARTICLE SIZE:

Table no: 03 – Particle Size

Batch code	Particle size in nm
F1	110.2
F2	307.1
F3	317.1
F4	251.8
F5	318.4

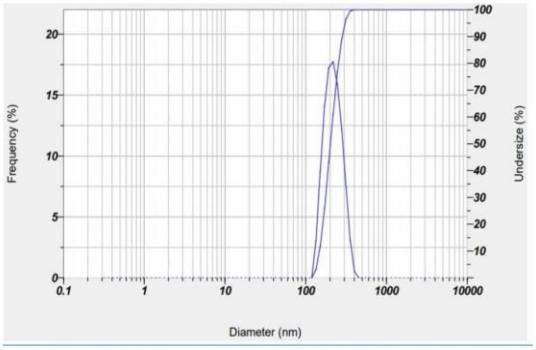

Figure no: 07 – Particle Size

ZETA POTENTIAL:

Table no: 04 – Zeta Potential

Batch code	Zeta potential (mV)
F1	-68.9
F2	-70.4
F3	-72.5
F4	-79.0
F5	-78.4

POLYDISPERSITY INDEX:

Table no: 05 – Polydispersity Index

Sample	Percentage (%)
F1	3.49
F2	2.79
F3	2.55
F4	2.15
F5	2.68

DRUG ENTRAPMENT EFFICIENCY:

Table no: 06 – DEE

Batch code	DEE(%)
F1	86%
F2	80%
F3	90%
F4	96%
F5	86%

DRUG CONTENT:

Table no: 07 – Drug Content

Formulation code	% Drug content
F1	61 ± 0.12%
F2	86± 0.17%
F3	53± 0.13%
F4	98 ± 0.11%
F5	90±0.10%

➢ **Microscopic evaluation:**

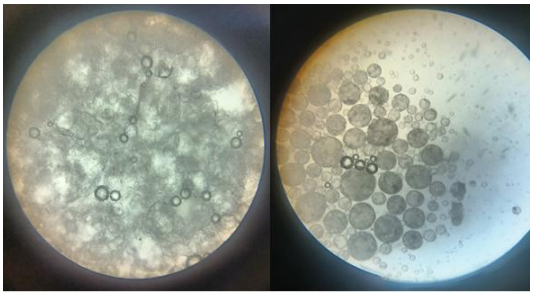

Figure no: 09 – Microscopic Evaluation

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IN-VITRO DIFFUSION:

Table no. 8: *In-Vitro* Diffusion Study

Time(hours)	F1	F2	F3	F4	F5
0	0	0	0	0	0
1	08 ± 0.14	09 ± 0.16	06 ± 0.23	13 ± 0.15	04± 0.11
2	16 ± 0.21	29 ± 0.14	14 ± 0.06	38 ± 0.26	16 ±0.14
3	32 ± 0.17	37 ± 0.22	35 ± 0.21	65 ± 0.19	25 ± 0.22
4	46 ± 0.32	45 ± 0.11	49 ± 0.19	70 ± 0.24	37 ± 0.14
5	59 ± 0.14	60 ± 0.18	58 ± 0.16	78 ± 0.28	44± 0.24
6	70 ± 0.13	78 ± 0.27	78 ± 0.31	84 ± 0.12	57± 0.32

STABILITY STUDY:

Table no: 9 – Stability Study

Parameters	Optimized batch (0 Month)	1 Month	2 Month	3 Month
Particle size (nm)	251.8	252.2	252.6	252.8
Zeta Potential (mV)	-79.0	-78.8	-78.6	-78.4
Polydispersity Index (PDI)	2.15%	2.16	2.18	2.19
Drug Entrapment Efficacy (%)	96%	95.8%	95.6%	95.4%

CONCLUSION:

Cubosomes prepared in dispersion possess a nanometre scale structure identical to bulk cubic phase, but the dispersion itself has much lower, water like viscosity. Compared to liposomes or vesicles cubosomes possess much higher viscous resistance to rupture. Cubosomes are being widely explored and are attracting a lot of attention, especially in preclinical investigations, because of their enticing features that are regarded as perfect for successful drug administration. Solubilisation of poor water-soluble medicines, along with the regulated and prolonged release of loaded actives, are two primary advantages of their use as delivery carriers. Cubosomes can be administered in a variety of ways, including intravenous, intranasal, oral, ophthalmic, and topical routes, due to their excellent qualities. Cubosome dispersions are convenient for using as oral, transdermal and parenteral drug delivery systems. Even though there is no well-known commercialized cubosome product yet, a significant number of publications have presented cubosomes as attractive pharmaceutical delivery system. Cubosomes prepared in dispersions possess a nanometre scale structure identical to bulk cubic phase, but the dispersion itself has much lower, water like viscosity. Compared to liposomes or vesicles, cubosomes possess much higher bilayer area-to-particle volume ratios as well as higher viscous resistance to rupture. Cubosomes are among a special class of lipid-based nanovesicles which characterized by liquid crystalline nature of their nanostructure, prepared from amphiphilic lipid which self-assembled in water and in presence of stabilizer into cubosomes. Although bulk cubic phase has sufficient length scale to allow controlled release of solutes, cubosomes are too small and have too high a surface area for such performance, exhibiting instead burst release. The main applications of cubosomes are controlled release of various drugs, in melanoma (cancer) therapy, oral drug delivery systems, intravenous drug delivery systems and topical drug delivery systems.

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