

INVENTION DISCLOSURE

Plant Extracellular Vesicle-Integrated CsA-Phospholipid Prodrug Platform for PLA2-Activated Oral Drug Delivery

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1 Executive Summary

1.1 One-Line Description

A pharmaceutical composition in which cyclosporine A (CsA)-phospholipid prodrugs with phospholipase A2 (PLA2)-cleavable sn-2 ester bonds are integrated directly into the membranes of plant-derived extracellular vesicles (PDEVs), enabling dual-level control of drug release: GI protection and tissue targeting via PDEVs, plus inflammation-triggered CsA activation via PLA2 cleavage.

1.2 Key Innovation

This invention combines two previously **separate** therapeutic strategies into a single, integrated platform:

1. **CsA-phospholipid prodrugs with PLA2-cleavable bonds** (validated technology from prior colon-targeted drug delivery literature)
2. **Plant-derived extracellular vesicles as oral carriers** (emerging biocompatible platform with GI stability and natural gut tropism)

The result is a **membrane-integrated prodrug delivery system** where:

- **Level 1 (Targeting & Protection):** PDEVs protect the integrated prodrugs through stomach/small intestine and accumulate in inflamed gut tissue via natural tropism
- **Level 2 (Activation):** Elevated PLA2 in diseased mucosa (10–100-fold increase in IBD) cleaves sn-2 ester bonds, releasing active CsA locally

This **dual-control mechanism** maximizes local immunosuppression while minimizing systemic exposure—addressing the core challenge of CsA therapy in inflammatory bowel disease (IBD).

1.3 Differentiation from Prior Art

Aspect	Prior Art	Our Invention
CsA-phospholipid prodrugs	Free prodrugs without protective carrier; require separate delivery vehicle	Integrated into plant EV membranes for GI protection and enhanced targeting
Plant EV carriers	Used for passive drug loading (hydrophobic molecules in bilayer)	Used as scaffold for prodrug integration , combining carrier function with enzyme-activated release
Targeting mechanism	Prodrugs alone: PLA2 activation but poor GI stability; Plant EVs alone: targeting but no activation trigger	Dual mechanism: PDEV targeting + PLA2 activation (synergistic)
Drug loading	Free prodrugs (no carrier) OR passive encapsulation in liposomes/EVs	Covalent/stable membrane integration of prodrug lipids into native plant EV lipid bilayer

Table 1: Comparison with existing CsA drug delivery technologies.

1.4 Target Applications

Primary indication: Acute severe ulcerative colitis (ASUC) – 75,000 cases/year in US/EU requiring rescue therapy.

Secondary indications:

- Crohn's disease (moderate-to-severe)
- Graft-versus-host disease (GVHD)
- Organ transplant immunosuppression (if long-term safety demonstrated)

Platform potential: Extensible to other hydrophobic immunosuppressants (tacrolimus, sirolimus, everolimus) conjugated to PLA2-cleavable phospholipids, and to other enzyme-triggered systems (MMPs, cathepsins, elastases) integrated into plant EVs.

2 Background and Unmet Need

2.1 Cyclosporine A in Inflammatory Bowel Disease

Cyclosporine A (CsA) is a potent calcineurin inhibitor that blocks T-cell activation by preventing NFAT nuclear translocation and IL-2 transcription. In acute severe ulcerative colitis (ASUC), intravenous CsA achieves 60–80% response rates as rescue therapy for patients failing high-dose corticosteroids. However, clinical utility is severely limited by:

- **Nephrotoxicity:** 20–40% of patients develop acute kidney injury (serum creatinine elevation >0.3 mg/dL)
- **Neurotoxicity:** Tremor, paresthesias, seizures in 5–10%
- **Narrow therapeutic window:** Blood levels must be maintained at 200–400 ng/mL (higher = toxicity; lower = inefficacy)
- **Pharmacokinetic variability:** Oral bioavailability: 20–50%; food effects; CYP3A4/P-glycoprotein interactions

Current oral formulations (Neoral, Gengraf) are microemulsions that improve absorption but do **not** achieve:

- Gut-specific targeting (systemic exposure remains high)
- Sustained local release (rapid clearance from inflamed tissue)
- Disease-responsive activation (drug released in healthy and diseased tissue equally)

2.2 Existing Prodrug and Carrier Strategies

2.2.1 PLA2-Cleavable Phospholipid Prodrugs (Prior Art)

Secreted phospholipase A2 (sPLA2, especially group IIA) is upregulated 10–100-fold in inflamed intestinal mucosa in IBD. CsA-phospholipid conjugates with sn-2 ester bonds have been developed as colon-targeted prodrugs in prior literature:

Key reference: “PLA2 Triggered Activation of Cyclosporine-Phospholipid Prodrug as a Drug Targeting Approach in Inflammatory Bowel Disease Therapy” and related publications.

Validated prodrug features:

- CsA conjugated to phospholipid via sn-2 ester linkage (e.g., via succinate or other linker)
- CsA remains inactive (low membrane permeability, low calcineurin binding) until PLA2 cleaves the lipid
- Selective activation in diseased tissue where sPLA2 is elevated
- Reduced systemic toxicity in animal models

Limitations of free prodrugs:

- Still requires oral delivery vehicle for GI protection (prodrugs are phospholipids, susceptible to gastric acid and proteases)

- Poor oral bioavailability without formulation
- Rapid clearance from intestinal lumen
- No intrinsic targeting mechanism (relies solely on enzyme distribution)

2.2.2 Plant-Derived Extracellular Vesicles (PDEVs)

PDEVs are naturally occurring nanoparticles (50–300 nm) secreted by plant cells. Citrus, grapefruit, ginger, and grape EVs have been shown to:

- Survive gastric pH and proteolytic enzymes
- Cross intestinal epithelial barriers (M-cell transcytosis, macropinocytosis)
- Deliver bioactive cargo (lipids, proteins, nucleic acids) to mammalian cells
- Exhibit anti-inflammatory properties (citrus EVs reduce colitis severity in mice)
- Have low immunogenicity and GRAS (Generally Recognized As Safe) status potential

Limitations of PDEVs alone:

- Passive drug loading only (limited to hydrophobic molecules in membrane or aqueous core)
- No controlled or triggered release mechanism
- Batch-to-batch variability (natural source)

2.3 Gap in the Field

No prior work has integrated CsA-phospholipid prodrugs into plant EV membranes. Each technology addresses one aspect of the problem:

- CsA-phospholipid prodrugs = enzyme-triggered activation
- PDEVs = GI protection + targeting + biocompatibility

Our invention **combines both**, creating a dual-level control system where:

1. The prodrug lipid is **incorporated into the PDEV membrane** (not free in solution), providing structural integration and stability
2. The PDEV carrier provides GI protection, enhanced oral bioavailability, and preferential accumulation in inflamed tissue
3. PLA2 activation releases active CsA locally at disease sites
4. The system leverages **validated prodrug chemistry** (reducing technical risk) while adding a **novel carrier integration** approach

3 Detailed Invention Description

3.1 Inventive Concept: Plant EV-Integrated Prodrug Platform

3.1.1 System Overview

The invention is a **membrane-integrated prodrug delivery platform** with the following structure:

1. **Prodrug Component:** Cyclosporine A conjugated to a phospholipid via PLA2-sensitive sn-2 ester bond
 - Structure: 1-acyl-2-(CsA-linker)-sn-glycero-3-phosphocholine (or similar)
 - sn-1 position: Long-chain fatty acid (C16–C18, e.g., palmitoyl) for membrane insertion
 - sn-2 position: CsA conjugated via cleavable ester linker (e.g., succinate, glutarate, or homoserine spacer)
 - sn-3 position: Phosphocholine or phosphoethanolamine head group
 - Design based on validated PLA2-cleavable prodrug literature
2. **Carrier:** Plant-derived extracellular vesicles (100–300 nm diameter)
 - Source: *Citrus sinensis* (sweet orange), *Citrus paradisi* (grapefruit), or other edible plants
 - Isolated via differential centrifugation + size-exclusion chromatography or tangential flow filtration
 - Characterized by: CD63, TSG101 (EV markers); size, PDI, zeta potential
 - Prodrug lipids are **integrated directly into PDEV bilayer membrane** via co-incubation or membrane fusion techniques

3.1.2 Dual-Level Mechanism of Action

Level 1 – GI Protection and Tissue Targeting (PDEV Function):

- After oral administration, PDEVs (with integrated prodrugs) provide protection from:
 - Acidic gastric pH (PDEVs are stable at pH 1.2 for >2 hours)
 - Proteolytic enzymes (pepsin, trypsin)
 - Premature prodrug degradation in stomach/small intestine
- PDEVs accumulate preferentially in inflamed intestinal tissue via:
 - Enhanced permeability (disrupted epithelial barrier in IBD)
 - M-cell transcytosis in Peyer's patches
 - Macrophage/dendritic cell uptake in lamina propria
 - Natural plant EV tropism for gut-associated lymphoid tissue (GALT)
- The integrated prodrug lipids remain stable in PDEV membranes during transit

Level 2 – Inflammation-Triggered Release (PLA2 Activation):

- In healthy tissue: Low PLA2 activity → prodrug lipids remain intact → CsA stays inactive
- In inflamed tissue: Elevated sPLA2-IIA (10–100-fold increase in IBD mucosa) → cleaves sn-2 ester bond → releases active CsA locally
- This creates spatial selectivity: CsA is released preferentially where disease is active and sPLA2 is abundant
- Cleavage products:
 - Free CsA (active immunosuppressant)
 - Lysophospholipid (1-acyl-lysophosphatidylcholine, biologically inert or mildly bioactive)
 - Both products can partition into local tissue or be taken up by immune cells

3.1.3 Synergistic Advantages

Challenge	How PDEV-Integrated Prodrug Addresses It
CsA poor oral bioavailability	PDEV encapsulation + membrane integration → improved GI stability and cellular uptake
Systemic toxicity (kidney, CNS)	Dual targeting (PDEV tropism + PLA2 activation) → preferential local release in inflamed gut, minimal systemic exposure
Free prodrug instability	Integration into PDEV membrane → protection from degradation, enhanced circulation time
Non-specific drug delivery	PLA2 activation → drug released preferentially at inflamed sites with elevated enzyme
GI instability of free prodrugs	PDEV shell → protects prodrug lipids through stomach acid and proteases
Batch variability of plant EVs	Standardized prodrug incorporation method → consistent CsA loading; EV isolation protocols validated in literature

Table 2: Synergistic problem-solving via PDEV-integrated prodrug platform.

3.2 Technical Feasibility and Prior Art Acknowledgment

Building on Validated Chemistry:

- CsA-phospholipid prodrugs with PLA2-cleavable bonds are **not novel per se**—they are described in prior publications on IBD-targeted drug delivery
- **Our novelty** is the integration into plant EV membranes as a delivery carrier, which:
 - Improves oral bioavailability (EV protection)
 - Enhances tissue targeting (EV tropism)
 - Reduces systemic exposure (dual-level control)
 - Leverages natural, food-derived carriers (GRAS status potential)

- This is a **carrier innovation** building on established prodrug chemistry, not a claim to invent PLA2-cleavable CsA prodrugs

Technical Risk Mitigation:

- Prodrug synthesis: Can be performed in-house or outsourced to contract chemistry labs (Avanti Polar Lipids, ChemCruz, etc.) for \$5K–10K per variant
- PDEV isolation: Established protocols from Dr. Herrera's lab (ExoVitaе) and literature
- Membrane incorporation: Co-incubation methods validated for lipid insertion into mammalian and synthetic liposomes; adaptation to plant EVs is straightforward
- PLA2-triggered release: Mechanism validated in prior prodrug studies; we are applying it to PDEV-integrated system

4 Detailed Manufacturing Method

4.1 Step 1: CsA-Phospholipid Prodrug Synthesis

4.1.1 Design Strategy

Target Structure: 1-Palmitoyl-2-(CsA-succinyl)-sn-glycero-3-phosphocholine (or similar with PLA2-cleavable sn-2 ester)

Key Features:

- sn-1: Palmitoyl (C16:0) or stearoyl (C18:0) for stable membrane insertion
- sn-2: CsA conjugated via succinate or glutarate spacer to sn-2 hydroxyl (forms ester bond cleavable by PLA2)
- sn-3: Phosphocholine head group for aqueous solubility and membrane compatibility
- Spacer optimization: Test 2–4 carbon linkers (succinate, glutarate) to balance PLA2 cleavage kinetics vs. stability

4.1.2 Synthesis Route (Outsourced or In-House)

Option A: Contract Synthesis (Preferred for Phase I)

- Contact: Avanti Polar Lipids, ChemCruz, or similar specialty lipid chemistry labs
- Provide CsA + desired phospholipid scaffold + linker specifications
- Request: 3–5 prodrug variants with different sn-1 fatty acids and sn-2 linker lengths
- Cost: \$5,000–10,000 per variant (5–50 mg scale)
- Timeline: 8–12 weeks
- Quality control: ^1H -NMR, ^{13}C -NMR, MS, HPLC purity $\geq 95\%$

Option B: In-House Synthesis (for Experienced Medicinal Chemists)

1. Start with 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (lysophosphatidylcholine, commercially available)
2. Activate sn-2 hydroxyl with succinic anhydride or glutaric anhydride \rightarrow forms dicarboxylic acid at sn-2
3. Activate carboxylic acid with EDC/NHS or DCC coupling agents
4. Couple with CsA (has free hydroxyl groups) under anhydrous conditions
5. Purify by silica gel chromatography or preparative HPLC
6. Characterize by NMR, MS, HPLC

Characterization:

- Structure confirmation: ^1H -NMR (characteristic CsA signals at 0.8–5.5 ppm + phospholipid peaks)

- Purity: HPLC-UV (single peak $\geq 95\%$)
- Mass: ESI-MS or MALDI-TOF (expected $m/z \sim 1,400\text{--}1,600$ depending on linker)
- PLA2 susceptibility: Incubate with recombinant sPLA2-IIA; quantify CsA release by HPLC over time (target: $>50\%$ release in 6h at physiological enzyme concentration)

4.2 Step 2: Plant EV Isolation

Source Material: Fresh-pressed juice from *Citrus sinensis* (sweet orange) or *Citrus paradisi* (grapefruit). Use organic, commercially available juice or fresh-squeeze.

Isolation Protocol (Differential Centrifugation + Size Exclusion Chromatography):

1. **Clarification:** Centrifuge juice at $500\times g$ (10 min) \rightarrow $2,000\times g$ (20 min) \rightarrow $10,000\times g$ (30 min, 4°C) to remove pulp, cells, and debris
2. **EV enrichment:** Ultracentrifuge supernatant at $100,000\times g$ (90 min, 4°C) to pellet EVs
3. **Purification:** Resuspend pellet in PBS; apply to qEV size-exclusion columns (Izon Science, 70 nm separation size) or equivalent
4. **Collection:** Collect fractions 7–9 (EV-enriched, excludes free proteins and small molecules)
5. **Concentration:** Use tangential flow filtration (TFF) with 100 kDa MWCO to concentrate to desired volume (target: 1–2 mg EV protein/mL)

Characterization:

- Particle concentration: NTA (target: $10^{10}\text{--}10^{11}$ particles/mL)
- Size: Mean diameter 100–200 nm, PDI < 0.3
- Morphology: TEM (negative stain with uranyl acetate; expect cup-shaped vesicles)
- EV markers: Western blot for CD63, TSG101 (enriched vs. crude juice)
- Protein content: BCA assay (yield: 50–200 μg protein per mL juice)
- Lipid content: Total lipid extraction + quantification (e.g., by Bligh-Dyer method)

4.3 Step 3: Prodrug Integration into PDEV Membranes

4.3.1 Method A: Co-Incubation (Preferred)

Rationale: Lipids can spontaneously insert into biological membranes via hydrophobic interactions. This method is gentle and preserves EV integrity.

Protocol:

1. Dissolve CsA-phospholipid prodrug in ethanol or DMSO (10 mM stock)
2. Add prodrug to purified PDEVs at defined molar ratios (prodrug:native PDEV lipids)

- Test ratios: 1:100, 1:20, 1:10, 1:5 mol:mol
 - Start with low ratios to avoid disrupting EV structure
3. Incubate at 37°C for 30–60 minutes with gentle mixing (rotator or orbital shaker, 10–20 rpm)
 4. **Optional:** Brief sonication (10–30 sec, low power) to facilitate membrane insertion (test if co-incubation alone is insufficient)
 5. Remove free (non-incorporated) prodrugs via:
 - Size-exclusion chromatography (qEV columns): EVs elute in fractions 7–9, free lipids in later fractions
 - OR ultracentrifugation (100,000×g, 90 min): pellet EVs, discard supernatant containing free prodrugs
 6. Resuspend purified PDEV-prodrug in PBS or storage buffer (10% trehalose for lyophilization)

4.3.2 Method B: Membrane Fusion / Extrusion (Alternative)

Protocol:

1. Prepare prodrug-containing liposomes (small unilamellar vesicles, SUVs):
 - Mix CsA-prodrug with helper lipids (e.g., DOPC, cholesterol) in organic solvent
 - Dry to form lipid film; rehydrate in PBS; extrude through 100 nm polycarbonate membrane
2. Mix prodrug-SUVs with PDEVs at defined ratios (1:1 to 1:10 mass ratio)
3. Induce membrane fusion via:
 - Freeze-thaw cycles (3–5 cycles, –80°C to 37°C)
 - OR calcium-induced fusion (add 1–5 mM CaCl₂, incubate 30 min, then remove Ca²⁺ by dialysis)
4. Purify as in Method A

4.3.3 Characterization of PDEV-Prodrug Hybrids

Prodrug Incorporation Quantification:

- Extract total lipids from purified PDEV-prodrug (Bligh-Dyer or Folch method)
- Quantify CsA content by HPLC or LC-MS/MS
- Calculate: mol% prodrug = (moles CsA-prodrug / total moles PDEV lipids) × 100
- Target: ≥5–10 mol% incorporation for therapeutic CsA doses

Structural Integrity:

- Size: DLS or NTA (expect 100–250 nm, slight increase vs. empty PDEVs acceptable)

- PDI: Should remain < 0.3 (polydisperse = aggregation or disruption)
- Zeta potential: May shift slightly due to prodrug insertion (monitor but expect –10 to –30 mV)
- TEM: Vesicles should retain cup-shaped morphology; no aggregation or membrane disruption
- EV markers: Western blot for CD63, TSG101 (should be retained, confirming EV integrity)

Stability Testing:

- **Storage stability:** Monitor CsA content, particle size, and PDI at 4°C (weekly for 4 weeks), –20°C, and –80°C
- **GI stability:** Incubate in simulated gastric fluid (SGF, pH 1.2, pepsin) for 2h, then simulated intestinal fluid (SIF, pH 6.8, pancreatin) for 6h. Quantify CsA retention and EV integrity.
- Target: ≥80% CsA retained, <20% change in particle size

4.4 Step 4: Final Formulation and Quality Control

Formulation Options:

- **Liquid suspension:** Store PDEV-prodrug at 4°C in PBS with 5–10% trehalose (cryoprotectant); use within 4 weeks
- **Lyophilized powder:** Freeze-dry with trehalose or sucrose (10–20% w/v); reconstitute before use. Advantages: long-term stability, ease of dosing
- **Enteric-coated capsules:** Fill lyophilized PDEV-prodrug into gelatin capsules; coat with Eudragit L100-55 or similar pH-sensitive polymer to protect through stomach (dissolves at pH > 5.5 in duodenum)

Release Specifications (for Preclinical/Clinical Development):

- CsA content: 90–110% of label claim (HPLC/LC-MS quantification)
- Particle size: 100–300 nm (NTA)
- Prodrug incorporation: ≥5 mol% of total PDEV lipids (LC-MS of lipid extract)
- Sterility: USP <71> (if for GMP production)
- Endotoxin: <5 EU/dose (LAL assay, relevant for clinical studies)
- Stability: ≥90% CsA retained at 4°C for 6 months (accelerated: 25°C for 3 months)

5 Patent Claims (Draft for Counsel)

5.1 Independent Claim 1: Composition of Matter

Claim 1. A pharmaceutical composition comprising:

- (a) plant-derived extracellular vesicles; and
- (b) cyclosporine A-phospholipid prodrugs integrated into a membrane of the plant-derived extracellular vesicles,

wherein the cyclosporine A-phospholipid prodrugs comprise a phospholipid moiety with a sn-2 ester bond that is cleavable by phospholipase A2 (PLA2), and wherein PLA2 activity releases cyclosporine A from the prodrug.

5.2 Dependent Claims (Examples)

Claim 2. The composition of Claim 1, wherein the plant-derived extracellular vesicles are isolated from a plant source selected from the group consisting of *Citrus sinensis*, *Citrus paradisi*, *Citrus limon*, *Zingiber officinale*, *Vitis vinifera*, and *Brassica oleracea*.

Claim 3. The composition of Claim 1, wherein the cyclosporine A-phospholipid prodrug has a structure:

- sn-1 position: a long-chain fatty acid (C14–C20);
- sn-2 position: cyclosporine A conjugated via a cleavable ester linker; and
- sn-3 position: a phosphocholine or phosphoethanolamine head group.

Claim 4. The composition of Claim 3, wherein the sn-1 fatty acid is palmitoyl or stearoyl.

Claim 5. The composition of Claim 3, wherein the sn-2 cleavable linker is a dicarboxylic acid selected from succinate, glutarate, and adipate.

Claim 6. The composition of Claim 1, wherein the cyclosporine A-phospholipid prodrugs comprise 5–30 mol% of the total lipid content of the plant-derived extracellular vesicles.

Claim 7. The composition of Claim 1, wherein the plant-derived extracellular vesicles have a mean diameter between 100 nm and 300 nm.

Claim 8. The composition of Claim 1, wherein the plant-derived extracellular vesicles retain at least 50% of endogenous plant bioactive compounds selected from flavonoids, polyphenols, and carotenoids.

Claim 9. The composition of Claim 1, wherein the composition is stable in simulated gastric fluid (pH 1.2) for at least 2 hours, retaining $\geq 80\%$ of cyclosporine A content.

Claim 10. The composition of Claim 1, wherein the sn-2 ester bond is selectively cleaved by secreted phospholipase A2 group IIA (sPLA2-IIA).

5.3 Independent Claim 2: Method of Treatment

Claim 11. A method of treating inflammatory bowel disease in a subject in need thereof, comprising orally administering to the subject a therapeutically effective amount of the composition of Claim 1,

wherein phospholipase A2 activity in inflamed intestinal tissue cleaves the sn-2 ester bond of the cyclosporine A-phospholipid prodrugs and releases cyclosporine A locally in the inflamed tissue.

Claim 12. The method of Claim 11, wherein the inflammatory bowel disease is selected from the group consisting of ulcerative colitis, acute severe ulcerative colitis (ASUC), Crohn's disease, and indeterminate colitis.

Claim 13. The method of Claim 11, wherein the oral administration achieves a plasma area-under-the-curve (AUC) of cyclosporine A that is less than 60% of the AUC achieved by an equivalent oral dose of free cyclosporine A.

Claim 14. The method of Claim 11, wherein the oral administration achieves a tissue concentration of cyclosporine A in the colon that is at least 2-fold higher than the tissue concentration achieved by an equivalent oral dose of free cyclosporine A.

Claim 15. The method of Claim 11, wherein the oral administration results in reduced nephrotoxicity compared to an equivalent dose of free cyclosporine A, as measured by serum creatinine or blood urea nitrogen levels.

Claim 16. The method of Claim 11, wherein the plant-derived extracellular vesicles provide protection of the cyclosporine A-phospholipid prodrugs from degradation in gastric acid and intestinal proteases.

5.4 Independent Claim 3: Method of Manufacture

Claim 17. A method of producing a plant extracellular vesicle-integrated cyclosporine A prodrug formulation, comprising:

- (a) synthesizing or obtaining a cyclosporine A-phospholipid prodrug comprising a sn-2 ester bond that is cleavable by phospholipase A2;
- (b) isolating extracellular vesicles from an edible plant material by differential centrifugation and size-exclusion chromatography or tangential flow filtration; and
- (c) contacting the cyclosporine A-phospholipid prodrugs with the extracellular vesicles under conditions that allow the prodrugs to integrate into a membrane of the extracellular vesicles,

thereby obtaining plant-derived extracellular vesicles with integrated cyclosporine A-phospholipid prodrugs.

Claim 18. The method of Claim 17, wherein the conditions for integrating the prodrugs into the extracellular vesicle membranes comprise incubation at 25–40°C for 30–120 minutes with gentle agitation.

Claim 19. The method of Claim 17, further comprising purifying the plant-derived extracellular vesicles with integrated prodrugs by size-exclusion chromatography or ultracentrifugation to remove non-integrated

prodrugs.

Claim 20. The method of Claim 17, wherein the prodrug incorporation efficiency is at least 5 mol% of the total lipid content of the extracellular vesicles.

5.5 Additional Claim Directions

Broadening to drug class:

- Replace "cyclosporine A" with "a hydrophobic calcineurin inhibitor selected from cyclosporine A, tacrolimus, pimecrolimus, and pharmaceutically acceptable salts thereof"
- Further broaden to "a hydrophobic immunosuppressant" (includes sirolimus, everolimus, mycophenolic acid derivatives)

Broadening to enzyme class:

- Replace "PLA2" with "an inflammation-associated enzyme selected from phospholipase A2, matrix metalloproteinase (MMP), cathepsin, and elastase"
- Design prodrugs with linkers cleavable by each enzyme class

Broadening to EV source:

- Expand beyond citrus to other edible plants: ginger, grapes, broccoli, carrots, turmeric
- Claim "plant-derived EVs from any edible plant material"

Additional use claims:

- Graft-versus-host disease (GVHD)
- Organ transplant rejection prevention
- Psoriasis, atopic dermatitis (topical or oral)
- Rheumatoid arthritis, lupus (oral systemic)

6 Prophetic Examples

6.1 Example 1: Synthesis of CsA-Phospholipid Prodrug

Procedure: A CsA-phospholipid prodrug with PLA2-cleavable sn-2 ester bond was synthesized as follows:

Starting materials:

- 1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (lysophosphatidylcholine, LPC; Avanti Polar Lipids)
- Succinic anhydride (Sigma-Aldrich)
- Cyclosporine A (Sigma C3662)
- Coupling reagents: EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide), NHS (N-hydroxysuccinimide)

Synthesis:

1. LPC (100 mg, 0.20 mmol) was dissolved in 10 mL anhydrous DMF
2. Succinic anhydride (30 mg, 0.30 mmol) was added under N₂ atmosphere
3. Triethylamine (50 μ L, 0.36 mmol) was added; reaction stirred at room temperature for 4h
4. Product (LPC-succinate dicarboxylic acid) was purified by silica gel chromatography
5. LPC-succinate (50 mg, 0.09 mmol) was activated with EDC (25 mg, 0.13 mmol) + NHS (15 mg, 0.13 mmol) in DMF for 30 min
6. Cyclosporine A (120 mg, 0.10 mmol) was added; reaction stirred overnight at room temperature
7. Product (CsA-succinate-LPC) was purified by preparative HPLC (reverse phase, C18 column, acetonitrile/water gradient)

Expected Results:

- Yield: 65–75% (85 mg product)
- Structure confirmation: ¹H-NMR (CDCl₃): CsA peaks at 0.8–5.5 ppm + LPC choline peak at 3.2 ppm + succinate linker at 2.6 ppm
- Mass: ESI-MS m/z = 1,805 [M+H]⁺ (calculated for C₉₂H₁₅₆N₁₁O₁₇P)
- Purity: HPLC-UV >96% (single peak at 18.5 min retention time)

PLA2 Susceptibility Test:

- Prodrug (100 μ M) incubated with recombinant sPLA2-IIA (1 μ g/mL) in PBS + 1 mM CaCl₂ at 37°C
- Aliquots taken at 0, 1, 3, 6, 24h; reaction quenched with EDTA
- Free CsA quantified by HPLC
- Expected: 12% release at 1h, 38% at 3h, 68% at 6h, 85% at 24h (confirming PLA2-mediated cleavage)

6.2 Example 2: Isolation and Characterization of Citrus EVs

Procedure: Fresh sweet orange juice (2 L, organic) was clarified by sequential centrifugation (500×g, 10 min; 2,000×g, 20 min; 10,000×g, 30 min). The supernatant was ultracentrifuged (100,000×g, 90 min, 4°C) to pellet EVs. The pellet was resuspended in PBS and purified by size-exclusion chromatography (qEV columns, Izon Science). Fractions 7–9 were collected and concentrated by tangential flow filtration (100 kDa MWCO).

Expected Results:

- Yield: 120 µg EV protein per mL juice (BCA assay)
- Particle concentration: 8×10^{10} particles/mL (NTA)
- Mean diameter: 165 ± 45 nm
- PDI: 0.27
- Morphology: TEM shows cup-shaped vesicles with intact bilayer membranes
- EV markers: Western blot positive for CD63, TSG101 (enriched 15-fold vs. crude juice)
- Bioactive cargo: HPLC detects hesperidin (32 µg/mg protein), naringenin (14 µg/mg protein)

Interpretation: Citrus EVs isolated with high purity, typical exosome-like characteristics, and retention of endogenous bioactive flavonoids.

6.3 Example 3: Integration of CsA-Prodrug into PDEV Membranes

Procedure: CsA-phospholipid prodrug (from Example 1) was dissolved in ethanol (10 mM stock). Prodrug solution (50 µL, 0.5 µmol) was added to purified citrus EVs (from Example 2, 1 mL, 1 mg protein/mL, ~10 µmol total PDEV lipids) to achieve a 1:20 mol:mol prodrug:PDEV lipid ratio. The mixture was incubated at 37°C for 45 min with gentle rotation (10 rpm). Free prodrugs were removed by size-exclusion chromatography (qEV columns); fractions 7–9 were collected (PDEV-prodrug).

Expected Results:

- Prodrug incorporation: 8.5 mol% of total PDEV lipids (LC-MS quantification of lipid extract)
- CsA content: 42 µg CsA per mg EV protein
- Size: 180 ± 55 nm (slight increase vs. empty EVs)
- Zeta potential: –18 mV (vs. –22 mV for empty EVs)
- TEM: EVs retain cup-shaped morphology; no aggregation
- Western blot: CD63, TSG101 retained (confirms EV integrity)

Interpretation: Successful integration of CsA-prodrug into PDEV membranes with high loading, preserved EV structure, and colloidal stability.

6.4 Example 4: PLA2-Triggered CsA Release from PDEV-Prodrug

Procedure: PDEV-prodrug hybrids (from Example 3) were incubated with: (1) PBS alone, (2) recombinant sPLA2-IIA (1 $\mu\text{g/mL}$, +1 mM CaCl_2), or (3) colon tissue homogenates from DSS-colitis mice (protein concentration normalized to 1 mg/mL). After 0, 1, 3, 6, 24 hours at 37°C, samples were centrifuged (10,000 \times g, 10 min) to separate released CsA (supernatant) from PDEV-bound prodrug (pellet). Free CsA in supernatant was quantified by HPLC.

Expected Results:

Condition	1h (% CsA release)	3h	6h	24h
PBS alone	5 \pm 2%	8 \pm 3%	12 \pm 3%	18 \pm 5%
sPLA2-IIA (1 $\mu\text{g/mL}$)	18 \pm 4%	42 \pm 6%	65 \pm 8%	82 \pm 10%
Inflamed tissue homogenate	22 \pm 5%	48 \pm 7%	70 \pm 9%	88 \pm 12%

Table 3: PLA2-triggered CsA release from PDEV-integrated prodrugs.

Interpretation: PLA2 activity (recombinant enzyme or from inflamed tissue) increases CsA release 3.5–5-fold vs. passive diffusion, confirming enzyme-responsive activation mechanism. Inflamed tissue homogenates show similar or higher release rates than purified enzyme, validating relevance to IBD pathophysiology.

6.5 Example 5: In Vitro T-Cell Suppression

Procedure: Human PBMCs were activated with anti-CD3/CD28 beads (Dynabeads, 1:1 bead:cell ratio) in 96-well plates (2 \times 10⁵ cells/well). Treatments were added simultaneously:

1. Vehicle (PBS)
2. Free CsA (50 ng/mL)
3. Free CsA-prodrug (50 ng/mL CsA-equivalent)
4. Empty PDEVs (equivalent EV dose)
5. PDEV-prodrug (25 ng/mL CsA-equivalent)

Some wells also received recombinant sPLA2-IIA (1 $\mu\text{g/mL}$) to mimic inflamed tissue. After 48h, supernatants were collected and IL-2 was measured by ELISA.

Expected Results:

Treatment	IL-2 (pg/mL)	% Suppression
Vehicle (activated, no drug)	1,250 \pm 150	–
Free CsA (50 ng/mL)	620 \pm 85	50%
Free CsA-prodrug (50 ng/mL)	980 \pm 120	22% (low activity, prodrug not activated)
Free CsA-prodrug + sPLA2	550 \pm 70	56% (activated by PLA2)
PDEV-prodrug (25 ng/mL), no PLA2	850 \pm 110	32% (partial activity from passive release)
PDEV-prodrug (25 ng/mL) + sPLA2	480 \pm 65	62% (superior, at half dose of free CsA)

Table 4: In vitro immunosuppressive activity with and without PLA2 activation.

Interpretation: In the presence of PLA2, the PDEV-integrated prodrug achieves superior suppression **at half the CsA dose** compared to free CsA, confirming: (i) enzyme-triggered activation, and (ii) enhanced cellular uptake via PDEV delivery. Without PLA2, PDEV-prodrug shows minimal activity, confirming prodrug-mediated inactivation until enzyme cleavage.

6.6 Example 6: GI Stability Testing

Procedure: PDEV-prodrug formulations (from Example 3) were exposed to:

1. Simulated gastric fluid (SGF): 0.1 M HCl + pepsin (3.2 mg/mL), pH 1.2, 37°C for 2h
2. Simulated intestinal fluid (SIF): 50 mM phosphate buffer + pancreatin (10 mg/mL), pH 6.8, 37°C for 6h

At each timepoint, aliquots were collected, neutralized, and analyzed for: (i) CsA content (HPLC), (ii) PDEV integrity (TEM, DLS), and (iii) prodrug incorporation (LC-MS of lipid extracts).

Expected Results:

- CsA retention after SGF (2h): $88 \pm 5\%$ (vs. $15 \pm 8\%$ for free CsA-prodrug)
- CsA retention after SIF (6h): $82 \pm 6\%$ (vs. $32 \pm 10\%$ for free prodrug)
- PDEV size: 185 ± 60 nm (minimal change from 180 nm initial)
- TEM: PDEVs remain intact; no membrane disruption
- Prodrug incorporation: 7.8 mol% (vs. 8.5% initial; 92% retention)

Interpretation: PDEV integration protects CsA-prodrug from harsh GI conditions (acidic pH, proteolytic enzymes), demonstrating crucial advantage over free prodrugs for oral delivery.

6.7 Example 7: In Vivo Efficacy and Safety in DSS-Colitis Model (Conceptual)

Study Design: C57BL/6 mice (n=10/group) receive 2.5% DSS in drinking water for 7 days to induce colitis. On days 3–7, mice are orally gavaged once daily with:

1. Vehicle (PBS)
2. Free CsA (10 mg/kg in microemulsion)
3. Free CsA-phospholipid prodrug (10 mg/kg CsA-equivalent)
4. Empty PDEVs (dose-matched to Group 5)
5. PDEV-integrated CsA-prodrug (5 mg/kg CsA-equivalent)

Expected Results:

Efficacy (Day 8):

- Disease activity index (DAI): Vehicle = 9.2 ± 1.3 ; Free CsA = 4.8 ± 0.9 ; Free prodrug = 6.5 ± 1.1 (poor efficacy, limited GI stability); **PDEV-prodrug = 3.8 ± 0.8** (58% reduction vs. vehicle, superior to free prodrug)
- Colon length: Vehicle = 5.2 cm; **PDEV-prodrug = 6.7 cm** (closer to healthy = 7.5 cm)
- Histology score: **PDEV-prodrug shows 52% lower inflammation score vs. vehicle**
- Colon tissue PLA2 activity: Inflamed mice show 18-fold higher sPLA2-IIA vs. healthy controls (confirming activation trigger is present)

Pharmacokinetics and Safety:

- Plasma CsA AUC_{0–6h}: Free CsA (10 mg/kg) = 3,800 ng·h/mL; **PDEV-prodrug (5 mg/kg) = 1,200 ng·h/mL** (68% lower systemic exposure despite superior efficacy)
- Colonic CsA concentration (terminal): Free CsA = 15 $\mu\text{g/g}$ tissue; Free prodrug = 8 $\mu\text{g/g}$; **PDEV-prodrug = 42 $\mu\text{g/g}$** (2.8-fold higher vs. free CsA; 5.3-fold vs. free prodrug)
- Serum creatinine: Vehicle = 0.24 mg/dL; Free CsA = 0.46 mg/dL (92% increase); **PDEV-prodrug = 0.27 mg/dL** (13% increase, not significant)
- Kidney histology: Free CsA shows moderate tubular vacuolation; **PDEV-prodrug shows minimal changes** (similar to vehicle)

Biodistribution (Fluorescent PDEV Tracking):

- Fluorescently labeled PDEVs (DiR dye) show preferential accumulation in inflamed colon (4.2-fold higher signal vs. healthy colon; 6.8-fold higher vs. kidney)
- Confirms PDEV-mediated tissue targeting

Interpretation: PDEV-integrated CsA-prodrug platform achieves superior therapeutic index—better efficacy at lower systemic exposure and no nephrotoxicity—validating dual-level control mechanism (PDEV targeting + PLA2 activation). Free prodrug without carrier shows limited efficacy due to poor GI stability, highlighting necessity of PDEV integration.

7 Commercial and Regulatory Strategy

7.1 Market Opportunity

Primary market: ASUC rescue therapy

- Epidemiology: 75,000 cases/year (US + EU)
- Current treatment: IV CsA (60–80% response) or infliximab (biologics, \$15K–20K/infusion)
- Unmet need: Safer oral CsA alternative to avoid colectomy (\$50K procedure + ICU stay)
- Pricing: \$3,000–5,000 per treatment course (7–14 days)
- Market size: \$50M–100M annually (conservative penetration)

Secondary markets:

- IBD maintenance therapy (if long-term safety demonstrated): \$500M+ opportunity
- GVHD (graft-versus-host disease): \$200M market for oral CsA alternatives
- Transplant immunosuppression: \$2B+ total CsA market

7.2 Regulatory Strategy

FDA Pathway: 505(b)(2) New Drug Application (NDA)

Rationale:

- CsA is approved (extensive safety/efficacy data exists from Neoral, Sandimmune)
- We are changing the delivery system (prodrug + plant EV carrier), not the active ingredient
- Can reference prior CsA approvals for some safety data
- Must demonstrate: (i) pharmaceutical equivalence (same drug, oral form), (ii) clinical superiority OR bioequivalence, (iii) safety of novel excipients (plant EVs = food-grade, GRAS status potential; prodrug = phospholipid, generally safe)

Development Timeline:

- **Phase I SBIR (Year 1):** Formulation optimization + preclinical POC (mice)
- **Phase II SBIR (Years 2–3):** GLP toxicology (28-day rodent + 90-day non-rodent) + CMC scale-up + IND preparation
- **Pre-IND meeting (Month 18):** FDA feedback on clinical trial design, CMC requirements, prodrug characterization
- **IND filing (Month 30):** Investigational New Drug application
- **Phase IIa clinical trial (Years 3–4):** 24 ASUC patients, single-arm, proof-of-concept (clinical response + PK)

- **Phase IIb (Years 4–5):** 120 patients, randomized, active-controlled (vs. standard IV CsA), non-inferiority + safety advantage
- **NDA filing (Year 6):** 505(b)(2) submission
- **Approval (Year 7):** Launch product

7.3 Intellectual Property Strategy

Patent Portfolio Plan:

1. **Provisional patent (Month 1):** File immediately with prophetic examples (no data required)
 - Title: “Plant Extracellular Vesicle-Integrated CsA-Phospholipid Prodrug Platform”
 - Claims: Composition (PDEV + integrated CsA-prodrug), treatment method, manufacturing process
 - Novelty focus: **Integration of validated prodrug into plant EV carrier** (not claiming to invent PLA2-cleavable prodrugs)
2. **Full utility patent (Month 12):** Convert provisional with real data from Phase I SBIR
 - Geographic coverage: US, EU (EPO), Canada, Japan, China, Brazil
 - Patent life: 20 years from filing = 2045 expiration (if filed 2025)
3. **Continuation-in-part (CIP, Year 2):** Add new claims based on Phase II data
 - Specific prodrug structures (after testing 3–5 variants)
 - Optimal incorporation methods (co-incubation conditions, ratios)
 - Clinical dosing regimens
4. **Use patent (Year 3):** Therapeutic method claims
 - Treatment of ASUC achieving <50% systemic exposure with equivalent efficacy
 - Combination therapy (PDEV-prodrug + biologics)

Freedom-to-Operate (FTO):

- **Prior art on CsA-phospholipid prodrugs:** Acknowledged; we are not claiming the prodrug chemistry itself, but the **carrier integration** (plant EVs)
- Preliminary search: No blocking patents identified for PDEV-integrated prodrug systems
- Watch list: Mammalian EV-prodrug combinations (different carrier type); synthetic liposome-prodrug formulations (different carrier origin)
- Our differentiation: **Natural plant EVs** (GRAS status, GI stability, intrinsic bioactivity) + **membrane integration method**

7.4 Licensing and Partnership Strategy

Target Partners:

- **Mid-size pharma with GI portfolio:** Takeda (Entyvio for UC), Ferring (European GI leader), AbbVie (Rinvoq, Skyrizi for IBD)
- **Generic pharma with specialty focus:** Dr. Reddy's, Amneal, Teva (seeking differentiated CsA products)

Deal Structure:

- **Stage 1 (After Phase I):** Option agreement (\$500K–1M for 12-month exclusive evaluation)
- **Stage 2 (After IND filing or Phase IIa):** Full license
 - Upfront: \$5M–15M (higher if clinical data available)
 - Milestones: \$50M–80M total (\$5M IND clearance, \$10M Phase IIa, \$15M Phase IIb, \$20M NDA approval, \$30M+ sales milestones)
 - Royalties: 5–10% of net sales (tiered)
- **Total deal value:** \$80M–120M (including milestones + early royalties)

Platform Licensing (Years 5–10): Beyond CsA, license the PDEV-integrated prodrug platform for:

- Tacrolimus-phospholipid prodrugs (GVHD, transplant)
- Sirolimus-phospholipid prodrugs (cancer, transplant)
- MMP-cleavable prodrugs (cancer, fibrosis)
- Cathepsin-cleavable prodrugs (cancer, inflammatory diseases)

Each new drug-enzyme pair = separate licensing deal (\$5M–10M per indication).

8 Conclusion

This invention establishes a **new paradigm in oral drug delivery** by integrating validated CsA-phospholipid prodrug chemistry into natural plant-derived extracellular vesicle carriers. The dual-level control mechanism—PDEV-mediated GI protection and tissue targeting plus PLA2-triggered local activation—addresses the fundamental challenge of delivering potent immunosuppressants like cyclosporine A to diseased tissue while minimizing systemic toxicity.

Key advantages:

- **Novel carrier integration:** First use of plant EVs as carriers for enzyme-activated prodrugs (no prior art identified for this specific combination)
- **Builds on validated chemistry:** CsA-phospholipid prodrugs have demonstrated PLA2-triggered activation; we add carrier-mediated protection and targeting
- **Superior therapeutic index:** Preclinical projections indicate >50% reduction in systemic exposure with equivalent or better efficacy
- **Scalable manufacturing:** Plant EVs from citrus juice waste (\$0.10–0.50/dose); established prodrug synthesis (contract labs available)
- **Regulatory advantage:** 505(b)(2) pathway (faster approval, lower cost than new molecular entity); GRAS status potential for plant EVs
- **Platform extensibility:** Applicable to multiple drugs (tacrolimus, sirolimus), enzymes (MMPs, cathepsins), and diseases (GVHD, transplant, cancer)

Novelty Statement for Patent Counsel:

- We acknowledge that CsA-phospholipid prodrugs with PLA2-cleavable bonds exist in prior literature
- **Our invention is the integration of these prodrugs into plant extracellular vesicle membranes**, which provides:
 - GI protection (plant EVs survive acidic stomach)
 - Enhanced oral bioavailability (EV-mediated cellular uptake)
 - Tissue targeting (natural EV tropism for inflamed gut)
 - Synergistic anti-inflammatory effects (plant EV bioactive cargo)
 - GRAS status pathway (food-derived carrier)
- This is a **composition and method of manufacture claim**, not a claim to the prodrug chemistry itself

We recommend:

1. **Immediate filing of provisional patent** to establish priority date
2. **SBIR Phase I application within 4–6 weeks** (with this disclosure as supporting material)

3. **Preliminary data generation (8–10 weeks):** Prodrug synthesis or procurement, PDEV isolation, membrane integration, in vitro PLA2-triggered release, cellular uptake and immunosuppression assays

This invention has the potential to transform treatment of inflammatory bowel disease and establish plant EV-based platforms as a commercially viable class of oral nanomedicines for enzyme-activated drug delivery.

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