INVENTION DISCLOSURE

Triple-Gated Plant Extracellular Vesicle–PLGA Nanoparticle Platform for Enzyme-Responsive Oral Drug Delivery

Inventors: Maria Beatriz Herrera Sanchez, PhD (Lead Inventor)
[Co-Inventor Name], PhD

Institution: ExoVitae Lab / [University/Company Name]

Date: November 23, 2025

Status: Confidential – For Patent Counsel Review

Contents

1 Executive Summary

1.1 One-Line Description

A multi-compartment oral drug delivery platform in which cyclosporine A (CsA)-loaded PLGA nanoparticles are encapsulated within plant-derived extracellular vesicles (PDEVs) and tethered via phospholipase A2 (PLA2)-cleavable lipid linkers, enabling triple-gated control of drug release: GI protection via PDEVs, inflammation-triggered nanoparticle liberation via PLA2 cleavage, and sustained local release via PLGA biodegradation.

1.2 Key Innovation

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

1. **PLGA nanoparticles for sustained CsA release** (established technology, widely used)
2. **Plant-derived extracellular vesicles as oral carriers** (emerging biocompatible platform)
3. **PLA2-cleavable phospholipid linkers for inflammation-triggered activation** (validated in colon-targeted prodrugs)

The result is a **"nested" architecture** where:

- **Level 1 (Targeting):** PDEVs protect PLGA NPs through stomach/small intestine and accumulate in inflamed gut tissue
- **Level 2 (Activation):** Elevated PLA2 in diseased mucosa cleaves lipid linkers, releasing PLGA NPs from PDEVs
- **Level 3 (Kinetics):** Liberated PLGA NPs provide sustained local CsA release over 24–72 hours

This **triple-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 formulations	Free drug, microemulsions, single-component nanoparticles (PLGA or liposomes)	**Nested NP-in-EV architecture** with enzyme-cleavable tethers
Carrier systems	Mammalian EVs OR plant EVs (not combined with synthetic NPs)	**Hybrid PDEV-PLGA system** exploiting benefits of both natural and synthetic carriers
Targeting mechanism	Passive accumulation OR surface ligands	**Enzyme-triggered release** exploiting disease-specific PLA2 upregulation
Release control	Single mechanism (diffusion or erosion)	**Triple-gated** (PDEV protection + PLA2 activation + PLGA kinetics)

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) and other enzyme-triggered release systems (MMPs, cathepsins, elastases).

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 Nanoformulation Strategies (Single-Level Control)

2.2.1 PLGA Nanoparticles

Poly(lactic-co-glycolic acid) is an FDA-approved biodegradable polymer widely used for sustained drug release. CsA-loaded PLGA NPs improve:

- Solubility (hydrophobic CsA encapsulated in polymer matrix)
- Release kinetics (hours to days, tunable via polymer MW and LA:GA ratio)
- Mucosal adhesion (some formulations)

Limitations:

- Poor GI stability (degradation in acidic stomach pH; enzymatic attack)
- Non-specific uptake (absorbed systemically and in healthy tissue)
- No intrinsic targeting or activation mechanism

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:

- Passive drug loading only (limited to hydrophobic molecules in membrane)
- No controlled release (drug diffuses out passively)
- Batch-to-batch variability (natural source)

2.2.3 PLA2-Cleavable Phospholipid Prodrugs

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:

- CsA remains inactive (locked) until PLA2 cleaves the lipid
- Selective activation in diseased tissue
- Reduced systemic toxicity

Limitations:

- Still requires oral delivery vehicle for GI protection
- Single-step activation (no sustained release after cleavage)
- Complex synthesis (low yields, high cost)

2.3 Gap in the Field

No prior work has combined PLGA NPs, plant EVs, and enzyme-cleavable linkers into a single platform. Each technology addresses one aspect of the problem:

- PLGA = kinetics control
- PDEVs = GI protection + targeting
- PLA2 linkers = activation trigger

Our invention **integrates all three**, creating a multi-level control system that is greater than the sum of its parts.

3 Detailed Invention Description

3.1 Inventive Concept: Triple-Gated Architecture

3.1.1 System Overview

The invention is a **nested nanoparticle-in-vesicle platform** with the following structure:

1. **Core:** Cyclosporine A encapsulated in PLGA nanoparticles (100–200 nm diameter)
    - PLGA composition: 50:50 to 75:25 lactide:glycolide ratio (MW 10–100 kDa)
    - CsA loading: 5–15 wt%
    - Release profile: 24–72 hours in vitro (phosphate buffer, pH 7.4, 37°C)
2. **Shell:** Plant-derived extracellular vesicles (150–300 nm diameter) encapsulating the PLGA NPs
    - 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
3. **Linker:** PLA2-cleavable phospholipid molecules inserted into the PDEV membrane
    - Structure: Phospholipid with sn-2 ester bond susceptible to sPLA2 cleavage
    - Function: Tether PLGA NPs to the inner surface of the PDEV membrane
    - Activation: PLA2 cleavage releases PLGA NPs from PDEV confinement

3.1.2 Triple-Gated Mechanism of Action

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

- After oral administration, PDEVs protect internal PLGA NPs from:
    - Acidic gastric pH (PDEVs are stable at pH 1.2 for $>$2 hours)
    - Proteolytic enzymes (pepsin, trypsin)
    - Premature drug release 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

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

- In healthy tissue: Low PLA2 activity $\rightarrow$ linkers remain intact $\rightarrow$ PLGA NPs stay confined in PDEVs

- In inflamed tissue: Elevated sPLA2-IIA (10–100-fold increase) → cleaves sn-2 ester bonds → liberates PLGA NPs from PDEV membrane
- This creates spatial selectivity: PLGA NPs are released preferentially where disease is active

Gate 3 – Sustained Local Release (PLGA Kinetics):

- Once liberated, PLGA NPs undergo biodegradation in the inflamed mucosa
- CsA is released gradually over 24–72 hours via:
  - Diffusion through polymer matrix (initial burst)
  - Polymer erosion (sustained phase)
  - pH-dependent hydrolysis (accelerated in acidic microenvironment of inflamed tissue)
- This sustains local immunosuppression without repeated dosing

3.1.3 Synergistic Advantages

Challenge	**How Triple-Gated System Addresses It**
CsA poor oral bioavailability	PLGA encapsulation + PDEV-mediated uptake → improved absorption
Systemic toxicity (kidney, CNS)	Triple-gated targeting + activation → preferential accumulation in gut, minimal systemic exposure
Short drug residence time in mucosa	PLGA sustained release → 24–72h local CsA levels
Non-specific drug delivery	PLA2 activation → drug released preferentially at inflamed sites
GI instability of nanoparticles	PDEV shell → protects PLGA NPs through stomach and small intestine

Table 2: Synergistic problem-solving via triple-gated control.

4 Detailed Manufacturing Method

4.1 Step 1: CsA-Loaded PLGA Nanoparticle Formulation

4.1.1 Method A: Nanoprecipitation (Preferred for Simplicity)

Materials:

- PLGA (50:50 LA:GA, MW 20–40 kDa, Sigma-Aldrich)
- Cyclosporine A (Sigma C3662 or equivalent)
- Organic solvent: Acetone or acetonitrile
- Stabilizer: Poloxamer 188 or polyvinyl alcohol (PVA, 1–2% w/v in water)

Protocol:

1. Dissolve PLGA (100 mg) + CsA (10–20 mg) in 5 mL acetone (organic phase)
2. Add dropwise to 20 mL aqueous phase (1% PVA) under magnetic stirring (500 rpm)
3. Continue stirring for 2–4 hours to evaporate organic solvent
4. Collect NPs by centrifugation (15,000$\times$g, 20 min) or ultrafiltration
5. Wash 2$\times$ with water to remove free CsA and stabilizer
6. Resuspend in PBS or trehalose solution (5% w/v)

Characterization:

- Size: NTA or DLS (target: 100–200 nm, PDI < 0.2)
- Zeta potential: Target: -10 to -30 mV (slightly negative for colloidal stability)
- CsA loading: Lyse NPs in acetonitrile, quantify CsA by HPLC or LC-MS/MS
- Encapsulation efficiency (EE%): (CsA in NPs / Total CsA added) $\times$ 100. Target: $\geq$60%
- In vitro release: Dialysis bag method (MWCO 50 kDa) in PBS pH 7.4 at 37°C; sample at 1, 6, 12, 24, 48, 72h

4.1.2 Method B: Double Emulsion (for Higher Loading)

Protocol:

1. **W1 phase:** Dissolve CsA (20 mg) in 1 mL ethanol
2. **O phase:** Dissolve PLGA (200 mg) in 10 mL dichloromethane (DCM)
3. **W1/O emulsion:** Add W1 to O dropwise under sonication (30 sec, 20% amplitude)
4. **W2 phase:** 40 mL aqueous PVA (2% w/v)
5. **W1/O/W2 emulsion:** Add W1/O to W2 under homogenization (10,000 rpm, 5 min)

6. Evaporate DCM under reduced pressure or stirring (4h, room temp)

7. Collect and wash NPs as above

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×g (10 min) → 2,000×g (20 min) → 10,000×g (30 min, 4°C) to remove pulp, cells, and debris

2. **EV enrichment:** Ultracentrifuge supernatant at 100,000×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

Characterization:

- Particle concentration: NTA (target: 10^{10}–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)

4.3 Step 3: PLA2-Cleavable Linker Synthesis and Incorporation

4.3.1 Linker Design

Concept: Use phospholipids with PLA2-sensitive sn-2 position as the cleavable element, adapted from validated colon-targeted prodrug designs.

Candidate Structure: 1-palmitoyl-2-(homoserinyl)-sn-glycero-3-phosphocholine or similar with:

- sn-1: Long-chain fatty acid (C16–C18) for membrane insertion
- sn-2: Ester bond linking to a functionalized moiety (e.g., homoserine) that can cross-link to PLGA NP surface
- sn-3: Phosphocholine head group for aqueous solubility

Synthesis: Custom synthesis by contract chemistry lab (e.g., Avanti Polar Lipids, ChemCruz) or in-house via standard phospholipid chemistry. Budget: $5,000–10,000 for 3–5 linker variants.

4.3.2 Incorporation into PDEVs

Method:

1. Prepare PLA2-cleavable lipids in ethanol or DMSO (10 mM stock)
2. Add to purified PDEVs (lipid:EV ratio: 1:100 to 1:10 mol:mol) under gentle mixing
3. Incubate at 37°C for 30–60 min to allow lipid insertion into EV membrane
4. Remove free lipids via SEC or ultracentrifugation
5. Confirm incorporation:
    - LC-MS: Detect linker lipid in PDEV lipid extract
    - TEM: No gross morphological changes
    - Zeta potential shift (linkers may alter surface charge)

4.4 Step 4: PLGA NP Encapsulation into PDEVs

Method A: Co-incubation with Mild Sonication

1. Mix CsA-PLGA NPs (1 mg/mL) with linker-functionalized PDEVs (1 mg protein/mL) at defined ratios (e.g., 1:1, 1:5, 1:10 NP:EV)
2. Incubate at 37°C for 1–2 hours under gentle rotation (10 rpm)
3. Apply brief bath sonication (30 sec, low power) to facilitate NP entry into EVs
4. Purify hybrids via density gradient centrifugation (e.g., OptiPrep or sucrose gradient) to separate NP-loaded EVs from free NPs and empty EVs

Method B: Extrusion

1. Mix NPs + PDEVs as above
2. Pass mixture through polycarbonate membrane filters (400 nm, 200 nm pore size) using mini-extruder
3. This forces NPs into EVs via mechanical shear
4. Purify as above

Characterization of PDEV-PLGA-CsA Hybrids:

- Size: Expect increase from 150 nm (empty PDEV) to 200–300 nm (loaded)
- NP encapsulation efficiency:
    - Quantitative: Flow cytometry (if NPs are fluorescently labeled)

  - Semi-quantitative: TEM imaging (count NPs per EV in >50 EVs)
  - Biochemical: Measure CsA content in purified hybrids; calculate CsA/EV protein ratio
- Structural integrity: TEM should show PLGA NPs inside PDEVs (not aggregated on surface)
- Stability: Monitor size and CsA content over 4 weeks at 4°C, -20°C, -80°C

4.5 Step 5: Final Formulation and Quality Control

Formulation:

- Add lyoprotectant (trehalose or sucrose, 5–10% w/v) to stabilize during storage or lyophilization
- For oral dosing:
  - Liquid suspension: Store at 4°C (use within 4 weeks) or -80°C (long-term)
  - Enteric-coated capsules: Lyophilize PDEV-PLGA-CsA hybrids; fill into capsules with enteric coating (Eudragit L100-55 or similar) to protect through stomach

Release Specifications (for clinical development):

- CsA content: 90–110% of label claim (HPLC/LC-MS)
- Particle size: 150–350 nm (NTA)
- Encapsulation efficiency: $\geq$40% of NPs associated with EVs (flow cytometry or TEM)
- Sterility: USP $<71>$ (if for GMP)
- Endotoxin: <5 EU/dose (LAL assay)
- Stability: $\geq$90% CsA retained at 4°C for 6 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;

(b) poly(lactic-co-glycolic acid) (PLGA) nanoparticles encapsulated within the plant-derived extracellular vesicles, wherein the PLGA nanoparticles comprise cyclosporine A; and

(c) phospholipid linkers associated with a membrane of the plant-derived extracellular vesicles,

wherein the phospholipid linkers are cleavable by phospholipase A2 (PLA2) and mediate association of the PLGA nanoparticles with the plant-derived extracellular vesicles such that PLA2 activity promotes release of the PLGA nanoparticles from the plant-derived extracellular vesicles.

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 PLGA nanoparticles have a mean diameter between 50 nm and 250 nm.

Claim 4. The composition of Claim 1, wherein the cyclosporine A loading in the PLGA nanoparticles is between 5 wt% and 20 wt%.

Claim 5. The composition of Claim 1, wherein the phospholipid linkers comprise a sn-2 ester bond that is selectively cleaved by secreted phospholipase A2 group IIA (sPLA2-IIA).

Claim 6. The composition of Claim 1, wherein the phospholipid linkers are 1-palmitoyl-2-(homoserinyl)-sn-glycero-3-phosphocholine or a structural analog thereof.

Claim 7. The composition of Claim 1, wherein the plant-derived extracellular vesicles have a mean diameter between 100 nm and 350 nm.

Claim 8. The composition of Claim 1, wherein the PLGA is a copolymer with a lactide:glycolide molar ratio between 50:50 and 85:15.

Claim 9. The composition of Claim 1, wherein the PLGA nanoparticles provide sustained release of cyclosporine A over a period of 24 to 72 hours in vitro in phosphate-buffered saline at pH 7.4 and 37°C.

Claim 10. 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.

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 phospholipid linkers and triggers release of the PLGA nanoparticles from the plant-derived extracellular vesicles into the inflamed tissue, resulting in sustained local cyclosporine A delivery.

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.

5.4 Independent Claim 3: Method of Manufacture

Claim 16. A method of producing a multi-compartment cyclosporine A formulation, comprising:

(a) preparing PLGA nanoparticles comprising cyclosporine A by nanoprecipitation or emulsion polymerization;

(b) isolating extracellular vesicles from an edible plant material by differential centrifugation and size-exclusion chromatography or tangential flow filtration;

(c) functionalizing the extracellular vesicles with phospholipid linkers comprising a sn-2 ester bond that is cleavable by phospholipase A2; and

(d) contacting the functionalized extracellular vesicles with the PLGA nanoparticles under conditions that allow the PLGA nanoparticles to be encapsulated within the extracellular vesicles and associated therewith via the phospholipid linkers,

thereby obtaining plant-derived extracellular vesicles containing cyclosporine A-loaded PLGA nanoparticles tethered by phospholipase A2-cleavable phospholipid linkers.

Claim 17. The method of Claim 16, wherein the conditions for encapsulating the PLGA nanoparticles within the extracellular vesicles comprise incubation at 37°C for 1–2 hours with gentle agitation, optionally followed by brief sonication or extrusion through a polycarbonate membrane.

Claim 18. The method of Claim 16, further comprising purifying the plant-derived extracellular vesicles containing the PLGA nanoparticles by density gradient centrifugation to separate nanoparticle-loaded vesicles from free nanoparticles and empty vesicles.

Claim 19. The method of Claim 16, wherein the encapsulation efficiency is at least 40%, defined as the percentage of PLGA nanoparticles associated with 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)

Broadening to polymer class:

- Replace ”PLGA” with ”a biodegradable polyester selected from poly(lactic-co-glycolic acid), polylactic acid, polycaprolactone, and copolymers thereof”

Broadening to enzyme class:

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

Additional use claims:

- Graft-versus-host disease (GVHD)
- Organ transplant rejection
- Psoriasis, atopic dermatitis (topical or oral)

6 Prophetic Examples

6.1 Example 1: CsA-Loaded PLGA Nanoparticle Formulation

Procedure: PLGA (50:50 LA:GA, MW 30 kDa, 100 mg) and cyclosporine A (15 mg) were dissolved in 5 mL acetone (organic phase). This solution was added dropwise to 20 mL aqueous solution containing 1% (w/v) polyvinyl alcohol (PVA) under magnetic stirring (500 rpm). After 4 hours of stirring to evaporate acetone, nanoparticles were collected by centrifugation (15,000×g, 20 min, 4°C), washed twice with water, and resuspended in PBS.

Expected Results:

- Mean diameter: 145 ± 25 nm (NTA)
- Polydispersity index (PDI): 0.18 ± 0.03
- Zeta potential: -18 ± 4 mV
- CsA loading: 12.5 wt% (HPLC quantification)
- Encapsulation efficiency: 83%
- In vitro release: 15% at 1h (burst), 45% at 24h, 78% at 72h (cumulative)

Interpretation: PLGA NPs with clinically relevant size, high CsA loading, and sustained release profile suitable for colon-targeted delivery.

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: PLA2-Cleavable Linker Incorporation

Procedure: Custom-synthesized 1-palmitoyl-2-(homoserinyl)-sn-glycero-3-phosphocholine (PLA2-sensitive linker) was dissolved in ethanol (10 mM stock). Linker (50 μL) was added to purified citrus EVs (1 mL, 1 mg protein/mL) and incubated at 37°C for 45 min with gentle mixing. Free linkers were removed by size-exclusion chromatography. Incorporation was confirmed by LC-MS analysis of EV lipid extracts.

Expected Results:

- Linker incorporation: 3.5% of total EV phospholipids (LC-MS quantification)
- Size: 170 ± 50 nm (slight increase vs. unmodified EVs)
- Zeta potential shift: from -22 mV to -15 mV (linker adds positive charge)
- TEM: No gross morphological changes; EVs remain intact

Interpretation: Successful insertion of PLA2-sensitive linkers into EV membranes without disrupting structural integrity.

6.4 Example 4: PLGA NP Encapsulation into PDEVs

Procedure: CsA-PLGA NPs (from Example 1, 1 mg/mL) were mixed with linker-modified citrus EVs (from Example 3, 1 mg protein/mL) at 1:5 NP:EV mass ratio. The mixture was incubated at 37°C for 2 hours under gentle rotation (10 rpm), followed by brief bath sonication (30 sec, 20% power). PDEV-PLGA hybrids were purified by OptiPrep density gradient centrifugation (collecting the fraction at 1.10–1.15 g/mL density).

Expected Results:

- Mean diameter: 285 ± 65 nm (NTA; increased from 165 nm for empty EVs)
- NP encapsulation efficiency: 52% (flow cytometry with fluorescent NPs)
- TEM: 2–5 PLGA NPs visible inside each EV; NPs appear tethered to inner membrane
- CsA content: 8 μg CsA per mg EV protein
- Stability: $>$85% of CsA retained at 4°C for 4 weeks; size remains stable

Interpretation: Successful formation of PDEV-PLGA-CsA hybrids with reproducible NP loading and colloidal stability.

6.5 Example 5: PLA2-Triggered NP Release

Procedure: PDEV-PLGA-CsA hybrids (from Example 4) were incubated with: (1) PBS alone, (2) recombinant sPLA2-IIA (1 μg/mL), or (3) colon tissue homogenates from DSS-colitis mice (protein concentration

normalized). After 0, 1, 3, 6 hours, samples were centrifuged (10,000×g, 10 min) to separate released NPs (supernatant) from intact PDEV-PLGA complexes (pellet). PLGA NPs in supernatant were quantified by fluorescence (if labeled) or HPLC (CsA content).

Expected Results:

Condition	1h (% NP release)	3h	6h
PBS alone	$8 \pm 2\%$	$12 \pm 3\%$	$18 \pm 4\%$
sPLA2-IIA (1 μg/mL)	$28 \pm 5\%$	$52 \pm 7\%$	$68 \pm 9\%$
Inflamed tissue homogenate	$35 \pm 6\%$	$58 \pm 8\%$	$72 \pm 10\%$

Table 3: PLA2-triggered PLGA NP release from PDEV-PLGA-CsA hybrids.

Interpretation: PLA2 activity (recombinant enzyme or from inflamed tissue) increases NP release 3–4-fold vs. passive diffusion, confirming enzyme-responsive activation mechanism.

6.6 Example 6: 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 (2×10^5 cells/well). Treatments were added simultaneously: (1) vehicle, (2) free CsA (50 ng/mL), (3) CsA-PLGA NPs (50 ng/mL CsA-equivalent), (4) PDEV-PLGA-CsA without cleavable linkers (50 ng/mL CsA), (5) PDEV-PLGA-CsA with PLA2-cleavable linkers (25 ng/mL CsA). Some wells also received recombinant sPLA2-IIA (1 μg/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 vs. vehicle
Vehicle (activated, no CsA)	$1{,}250 \pm 150$	–
Free CsA (50 ng/mL)	620 ± 85	50%
CsA-PLGA NPs (50 ng/mL)	550 ± 70	56%
PDEV-PLGA-CsA, no linker (50 ng/mL)	680 ± 95	46%
PDEV-PLGA-CsA + PLA2 linker (25 ng/mL)	700 ± 100	44%
PDEV-PLGA-CsA + PLA2 linker (25 ng/mL) + sPLA2	480 ± 65	62%

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

Interpretation: In the presence of PLA2, the cleavable PDEV-PLGA-CsA formulation achieves superior suppression **at half the CsA dose** compared to free CsA or non-cleavable controls, confirming enzyme-triggered potentiation.

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. CsA-PLGA NPs (10 mg/kg CsA-equivalent)
4. Non-cleavable PDEV-PLGA-CsA (10 mg/kg)
5. PLA2-cleavable PDEV-PLGA-CsA (5 mg/kg CsA-equivalent)

Expected Results:

Efficacy (Day 8):

- Disease activity index (DAI): Vehicle = 9.2 $\pm$ 1.3; Free CsA = 4.8 $\pm$ 0.9; PLGA NPs = 4.2 $\pm$ 0.8; Non-cleavable PDEV-PLGA = 4.5 $\pm$ 0.9; **PLA2-cleavable PDEV-PLGA = 3.5 $\pm$ 0.7** (61% reduction vs. vehicle, superior to all controls)
- Colon length: Vehicle = 5.2 cm; **PLA2-cleavable PDEV-PLGA = 6.8 cm** (closer to healthy = 7.5 cm)
- Histology score: **PLA2-cleavable group shows 55% lower inflammation score vs. vehicle**

Pharmacokinetics and Safety:

- Plasma CsA AUC_{0-6h}: Free CsA (10 mg/kg) = 3,800 ng·h/mL; **PLA2-cleavable PDEV-PLGA (5 mg/kg) = 1,100 ng·h/mL** (71% lower systemic exposure despite superior efficacy)
- Colonic CsA concentration (terminal): Free CsA = 15 μg/g tissue; **PLA2-cleavable PDEV-PLGA = 48 μg/g** (3.2-fold higher local accumulation)
- Serum creatinine: Vehicle = 0.24 mg/dL; Free CsA = 0.46 mg/dL (92% increase); **PLA2-cleavable PDEV-PLGA = 0.28 mg/dL** (17% increase, not significant)
- Kidney histology: Free CsA shows moderate tubular vacuolation; **PLA2-cleavable PDEV-PLGA shows minimal changes** (similar to vehicle)

Interpretation: Triple-gated PDEV-PLGA-CsA platform achieves superior therapeutic index—better efficacy at lower systemic exposure and no nephrotoxicity—validating all three control mechanisms (PDEV targeting, PLA2 activation, PLGA release).

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, 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 (PLGA is FDA-approved; PDEVs = food-grade)

Development Timeline:

- **Phase I SBIR (Year 1):** Formulation optimization + preclinical POC (mice)
- **Phase II SBIR (Years 2–3):** GLP toxicology (28-day rodent + non-rodent) + CMC scale-up + IND preparation
- **Pre-IND meeting (Month 18):** FDA feedback on clinical trial design, CMC requirements
- **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: "Triple-Gated Plant Extracellular Vesicle–PLGA Nanoparticle Platform"
    - Claims: Composition (PDEV + PLGA + PLA2 linker), treatment method, manufacturing process
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 linker structures (after testing 3–5 variants)
    - Optimal PLGA formulations (LA:GA ratio, MW)
    - Clinical dosing regimens
4. **Use patent (Year 3):** Therapeutic method claims
    - Treatment of ASUC achieving $<$50% systemic exposure
    - Combination therapy (PDEV-PLGA-CsA + biologics)

Freedom-to-Operate (FTO):

- Preliminary search: No blocking patents identified for the specific combination of PLGA NPs + plant EVs + PLA2 linkers
- Watch list: Mammalian EV-PLGA hybrids (different carrier type); PLA2-prodrugs (different activation mechanism); plant EV carriers (single-level system)
- Our differentiation: **Triple-gated architecture** is novel; integration of three independent technologies

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 triple-gated platform for:

- Tacrolimus + MMP-cleavable linkers (GVHD, transplant)
- Cannabinoids + cathepsin-cleavable linkers (pain, GI motility disorders)
- Anti-inflammatory peptides + elastase-cleavable linkers (pancreatitis)

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 natural (plant EV) and synthetic (PLGA) nanotechnologies with molecular-level activation triggers (PLA2-cleavable linkers). The triple-gated control mechanism—GI protection, inflammation-responsive release, and sustained local kinetics—addresses the fundamental challenge of delivering potent immunosuppressants like cyclosporine A to diseased tissue while minimizing systemic toxicity.

Key advantages:

- **Novel architecture:** First integration of PLGA + plant EVs + enzyme-cleavable linkers (no prior art identified)
- **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 PLGA production
- **Regulatory advantage:** 505(b)(2) pathway (faster approval, lower cost than new molecular entity)
- **Platform extensibility:** Applicable to multiple drugs, enzymes, and diseases

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):** CsA-PLGA formulation, PDEV isolation, hybrid assembly, in vitro PLA2-triggered release

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.

Inventors' Signatures:

_____ Date: _____

Maria Beatriz Herrera Sanchez, PhD
ExoVitae Lab

_____ Date: _____

Co-Inventor Name
, PhD