

Advanced Therapeutic Platform: Electroporation-Mediated Delivery Of Desharpened OMV-Encoded Antibody Particles For Targeted Tumor Membrane Engineering

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Abstract—This comprehensive technical exposition describes a multimodal bioengineering approach combining bacterial outer membrane vesicles (OMVs), nucleic acid nanocarriers, and electroporation for in situ cancer immunotherapy. The proposed methodology employs genetically or chemically "desharpened" OMVs---bacterial-derived nanostructures with reduced endotoxic activity but retained fusogenic and immunomodulatory properties---as biocompatible carriers for nanoparticles encapsulating antibody-encoding genetic material. These OMV-particle complexes are administered via intratumoral injection followed by targeted electroporation, which simultaneously permeabilizes tumor cell membranes and facilitates electrophoretic delivery of the complexes into the cytoplasm. Subsequent translation of the encoded genetic cargo transforms tumor cells into autonomous biofactories capable of sustained, local production of therapeutic antibodies. This paper systematically deconstructs each platform component, from fundamental biological mechanisms through engineering challenges, preclinical validation strategies, translational pathways, and future innovation opportunities.

Keywords—*Electroporation; Outer Membrane Vesicles; Targeted Tumor Therapy; Antibody Delivery; Genetic Medicine; Biomedical Engineering; Cancer Immunotherapy*

Introduction

The Unmet Need in Solid Tumor Immunotherapy

The advent of immune checkpoint inhibitors and chimeric antigen receptor (CAR) T-cell therapies has revolutionized oncology, yet durable responses in solid tumors remain limited to a minority of patients. Key barriers include the immunosuppressive tumor microenvironment (TME), physical barriers to lymphocyte infiltration, antigen heterogeneity, and systemic toxicity associated with high-dose antibody administration. While local delivery of therapeutic antibodies offers theoretical advantages in terms of efficacy and safety, protein-based approaches suffer from rapid clearance, poor tissue penetration, and prohibitive manufacturing costs.

Evolution of Nucleic Acid Delivery Systems

Nucleic acid therapeutics, particularly mRNA vaccines, have demonstrated remarkable clinical success. However, the translation of these technologies to oncology has been constrained by inefficient delivery to solid tumors, requirement for repeated systemic administration, and insufficient duration of transgene expression. The ideal delivery system should provide protection from extracellular nucleases, facilitate cellular uptake, enable endosomal escape, and achieve sustained therapeutic levels while minimizing off-target effects.

Bacterial Outer Membrane Vesicles as Next-Generation Carriers

Gram-negative bacteria naturally secrete 20-250 nm spherical nanostructures derived from the outer membrane, termed outer membrane vesicles (OMVs). These vesicles possess intrinsic adjuvanticity, fusogenicity with mammalian membranes, and the capacity to carry diverse cargoes. Recent advances in synthetic biology have enabled the engineering of "desharpened" OMVs with attenuated endotoxin activity while preserving or enhancing their delivery capabilities. This positions OMVs as uniquely versatile carriers for cancer therapeutic delivery.

Electroporation: From Laboratory Tool to Clinical Modality

Electroporation---the application of controlled electric fields to transiently permeabilize cell membranes---has evolved from a laboratory technique for *in vitro* transfection to a clinically validated modality for DNA vaccination and intratumoral drug delivery (electrochemotherapy). Modern electroporation devices offer precise control over pulse parameters, real-time impedance monitoring, and electrode configurations adaptable to various tumor geometries.

The Convergence Paradigm: Rationale for Platform Integration

No single delivery technology has proven universally adequate for solid tumor nucleic acid therapy. Viral vectors face immunogenicity and manufacturing constraints; lipid nanoparticles demonstrate preferential hepatic tropism; and physical methods alone lack carrier-mediated protection and targeting. The OMV-electroporation hybrid platform addresses these gaps through spatial complementarity (electroporation drives local accumulation, OMVs facilitate membrane interaction), temporal synergy (immediate electrotransfer followed by sustained OMV-mediated expression), and biological cooperation (OMV immunostimulation potentiates antibody-mediated antitumor effects). This convergence creates a system where the whole exceeds the sum of its parts.

Fundamentals of Outer Membrane Vesicle Biology and Engineering

Biogenesis and Native Functions of OMVs

Outer membrane vesicles are constitutively produced by Gram-negative bacteria through blebbing of the outer membrane, a process influenced by peptidoglycan-membrane connections, envelope stress responses, and quorum sensing signals. Native OMVs contain outer membrane proteins, lipopolysaccharide (LPS), periplasmic components, and sometimes nucleic acids. Their biological functions include interbacterial communication, virulence factor delivery, and immune modulation of host organisms.

Immunogenicity and Toxicity Profiles

The potent immunostimulatory properties of OMVs derive primarily from pathogen-associated molecular patterns (PAMPs), particularly LPS, lipoproteins, and flagellin. While this adjuvanticity is advantageous for vaccination applications, it poses toxicity risks for therapeutic delivery. Systemic administration of native OMVs can elicit cytokine storm syndromes, limiting their clinical utility without modification.

Genetic and Chemical Strategies for OMV Desharpener

Desharpener refers to the attenuation of OMV-associated toxicity while retaining desirable delivery and immunomodulatory functions. Two principal approaches have emerged:

Genetic Desharpener: Engineering of bacterial production strains with modified LPS biosynthesis pathways. The *msbB* mutation eliminates myristoylation of lipid A, generating penta-acylated LPS with substantially reduced Toll-like receptor 4 (TLR4) agonism. PagL modification introduces deacylated lipid A species. Further refinements include the expression of "detoxified" LPS variants from non-pathogenic bacteria and the deletion of immunostimulatory protein antigens.

Chemical Desharpener: Post-isolation treatment of OMVs with detergents (e.g., deoxycholate) to extract a portion of lipid A, or enzymatic digestion of surface proteins. While effective at reducing pyrogenicity, chemical methods may compromise vesicle integrity and fusogenicity.

LPS Modification and Detoxification Approaches

The structure-activity relationship of lipid A dictates its immunological potency. Hexa-acylated, bis-phosphorylated lipid A (*E. coli*-type) is the most potent TLR4 agonist. Desharpening strategies aim to generate under-acylated, monophosphoryl, or otherwise structurally altered lipid A species. Monophosphoryl lipid A (MPLA), derived from *Salmonella minnesota* R595, retains TLR4 agonist activity sufficient for adjuvanticity but with substantially reduced reactogenicity. Engineering OMV production strains to incorporate MPLA-like lipid A species represents an optimal desharpening strategy.

Scalable Production and Purification Methodologies

Industrial-scale OMV production has been established for vaccine manufacturing (e.g., meningococcal B vaccines). Typical workflows include:

- Fermentation: High-density bacterial culture in defined media, with process controls for pH, dissolved oxygen, and nutrient feeding.
- Harvest and Concentration: Tangential flow filtration (TFF) or hollow fiber systems to concentrate culture supernatant.
- Purification: Sequential TFF with defined pore sizes, followed by density gradient ultracentrifugation (sucrose or iodixanol) or size exclusion chromatography.
- Sterilization: 0.22 μ m filtration (feasible for OMVs smaller than 220 nm) or aseptic processing.
- Formulation: Buffer exchange into pharmaceutically acceptable vehicles (e.g., histidine-sucrose, trehalose).

Characterization and Quality Control Parameters

Comprehensive OMV characterization requires orthogonal analytical methods:

- Physical: Nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), cryo-electron microscopy for size, morphology, and aggregation state.
- Biochemical: Protein content (BCA assay), LPS quantification (Limulus amoebocyte lysate assay with modified protocols for detoxified LPS), lipid profiling.

- Immunological: TLR4 reporter cell assays, cytokine induction in human peripheral blood mononuclear cells (PBMCs), pyrogen testing.

- Functional: Cellular uptake assays, membrane fusion quantification (lipid mixing assays), stability under physiological conditions.

Antibody-Encoding Genetic Cargo Systems

Selection of Antibody Targets for Tumor Microenvironment Modulation

The platform's versatility allows encoding of diverse antibody formats:

- Checkpoint Inhibitors: Anti-PD-1, anti-PD-L1, anti-CTLA-4 scFv or full-length IgG. Local production may overcome resistance mechanisms associated with systemic therapy.

- Tumor-Targeting Antibodies: Anti-HER2, anti-EGFR, anti-GD2 for direct induction of apoptosis or antibody-dependent cellular cytotoxicity (ADCC).

- Immunostimulatory Antibodies: Agonistic anti-CD40, anti-4-1BB, anti-OX40 to activate antigen-presenting cells and T cells within the TME.

- Bispecific T-cell Engagers (BiTEs): Single-chain constructs simultaneously binding tumor-associated antigens and CD3 ϵ , redirecting cytotoxic T cells irrespective of TCR specificity.

- Immunocytokines: Antibody-cytokine fusions (e.g., IL-2, IL-12, TNF α) targeting the TME while minimizing systemic cytokine toxicity.

Nucleic Acid Platforms: mRNA, saRNA, and Minicircle DNA

- mRNA: Synthetic, modified nucleoside-containing mRNA (e.g., N1-methylpseudouridine) with optimized 5' and 3' untranslated regions (UTRs), cap structures, and poly-A tails. Advantages: cytoplasmic translation, no nuclear barrier, no integration risk. Disadvantages: transient expression, cold chain dependence.

- Self-Amplifying RNA (saRNA): Derived from alphavirus genomes, encoding both the antibody gene and RNA-dependent RNA polymerase for intracellular

amplification. Enables prolonged expression with lower initial RNA doses.

- Minicircle DNA: Supercoiled DNA expression cassettes devoid of bacterial plasmid backbones. Reduced size and absence of unmethylated CpG motifs enhance transgene expression and reduce immunogenicity compared to conventional plasmids. Requires nuclear delivery.

- Conventional Plasmid DNA: Ease of manufacturing and stability; however, lower transfection efficiency and potential for bacterial DNA-mediated innate immune activation.

Nanoparticle Formulation Strategies for Cargo Protection

Encapsulation of nucleic acids within nanoparticles is essential for protection against serum nucleases and to facilitate OMV association. Primary platforms include:

- Lipid Nanoparticles (LNPs): Four-component systems (ionizable cationic lipid, phospholipid, cholesterol, PEG-lipid) that self-assemble around nucleic acids through electrostatic interactions and pH-dependent ionization. Clinically validated for siRNA and mRNA delivery.

- Polymeric Nanoparticles: Poly(lactic-co-glycolic acid) (PLGA), polyethylenimine (PEI), or cyclodextrin-based polymers. Offer tunable release kinetics and enhanced stability.

- Cationic Nanoemulsions: Oil-in-water emulsions stabilized by cationic surfactants, suitable for RNA encapsulation and demonstrated intramuscular delivery.

Cationic Lipid and Polymer Encapsulation Technologies

The selection of cationic lipid components critically influences particle performance:

- Ionizable Lipids: DLin-MC3-DMA (patented by Arbutus), SM-102 (Moderna), ALC-0315 (BioNTech/Pfizer). These lipids exhibit net cationic charge at acidic pH (facilitating RNA complexation during manufacturing) but remain neutral at

physiological pH (reducing nonspecific interactions and toxicity).

- Helper Lipids: DSPC or DOPE, promoting bilayer fluidity and endosomal fusion.

- PEG-Lipids: Controlling particle size and preventing opsonization, with trade-offs between stability and cellular uptake.

Stability, Release Kinetics, and Shelf-Life Considerations

LNP-RNA formulations require frozen storage (-20° C to -80° C) to maintain potency, though lyophilization strategies are emerging. Critical quality attributes include:

- Encapsulation Efficiency: $\geq 90\%$ desired, quantified by RiboGreen assays with/without detergent.

- Particle Size: 60-100 nm optimal; larger particles exhibit reduced tissue penetration; smaller particles show accelerated clearance.

- Polydispersity Index: < 0.2 indicating monodisperse population.

- In Vitro Release: Quantified under sink conditions using molecular beacons or radiolabeled RNA.

OMV-Particle Complex Engineering

Principles of Carrier-Cargo Association

The physical and functional association of OMVs with nucleic acid nanoparticles is central to platform function. Ideal complexes maintain OMV membrane integrity, protect encapsulated cargo from premature release, and facilitate coordinated cellular uptake. Four primary association mechanisms have been investigated.

Passive Adsorption and Electrostatic Complexation

Negatively charged OMVs (due to LPS phosphate groups and acidic membrane proteins) can associate with cationic LNP or polymeric particles through electrostatic attraction. Simple mixing with gentle agitation yields complexes with variable stability and cargo retention. Optimization parameters include ionic strength, pH, relative concentrations, and mixing order.

Chemical Conjugation and Covalent Linkage Strategies

Stable, covalent attachment of nanoparticles to OMV surfaces can be achieved through bioorthogonal chemistry:

- EDC/NHS Coupling: Activation of carboxyl groups on OMV surface proteins for amide bond formation with amine-functionalized nanoparticles.
- Click Chemistry: Metabolic incorporation of azide-modified sugars into OMV LPS, enabling copper-free cycloaddition with dibenzocyclooctyne (DBCO)-modified nanoparticles.
- Streptavidin-Biotin: Biotinylation of OMV surfaces (via NHS-biotin) followed by incubation with streptavidin-coated nanoparticles.

Membrane Insertion and Hydrophobic Anchoring

Lipophilic moieties (e.g., cholesterol, DSPE-PEG) conjugated to nanoparticles can insert spontaneously into OMV lipid bilayers. This approach mimics viral envelope incorporation strategies and preserves nanoparticle structural integrity while achieving membrane anchorage.

Characterization of Complex Integrity and Loading Efficiency

- Quantification: Radiolabeled or fluorescently labeled nanoparticles enable determination of particle-to-OMV ratios and association efficiency.
- Structural Analysis: Cryo-EM and tomographic reconstruction reveal spatial distribution of nanoparticles relative to OMV membrane (surface-bound, partially embedded, or internalized).
- Stability Assays: Resistance to dissociation upon heparin competition, serum exposure, or shear stress; cargo retention measured by nuclease protection assays.

Optimization of Particle-to-OMV Ratios

Excess nanoparticle loading risks OMV aggregation, membrane destabilization, and altered biological properties. Suboptimal loading reduces therapeutic potency. Systematic optimization employs design of experiments (DoE) methodologies with

response surface modeling to identify maximal functional loading without compromising OMV integrity or uptake.

Electroporation Physics and Biological Interactions Biophysical Principles of Membrane Electroporation

Application of an external electric field induces transmembrane potential difference ($\Delta \Psi_m$) proportional to field strength and cell radius. When $\Delta \Psi_m$ exceeds threshold values (approximately 200 mV⁻¹ V, depending on pulse duration), hydrophilic pores form in the lipid bilayer, providing conduits for ionic and macromolecular transport. The primary electropore population is nanometer-scale and transient, resealing over milliseconds to minutes depending on temperature and membrane composition.

Pulse Parameters: Voltage, Duration, Frequency, and Waveform

- Electric Field Strength (E): Determines spatial extent of permeabilization. Typical intratumoral electroporation uses 400-1500 V/cm for reversible permeabilization. Higher fields risk thermal damage and irreversible electroporation.
- Pulse Duration (τ): Microsecond to millisecond pulses. Shorter pulses (μ s) primarily permeabilize plasma membranes; longer pulses (ms) facilitate electrophoretic transport of nucleic acids.
- Pulse Number (n): Multiple pulses (4-8) increase cumulative permeabilization and molecular uptake.
- Frequency: Low frequency (1-5 Hz) allows membrane resealing between pulses; high frequency (kHz) bursts may alter pore dynamics.
- Waveform: Exponential decay (classical) versus square wave (modern). Square wave pulses provide precise control over field strength throughout pulse duration.

Electrode Configurations and Field Distribution Modeling

- Penetrating Electrodes: Needle arrays (two to seven needles) inserted into tumor tissue. Configurable spacing (4-10 mm) to encompass entire

tumor volume. Hexagonal arrays provide more homogeneous field distribution than parallel needles.

- Plate Electrodes: Non-penetrating, suitable for superficial cutaneous tumors. Field penetration depth limited to approximately half electrode spacing.
- Finite Element Modeling: Computational simulation of electric field distribution using patient-specific imaging data enables treatment planning and parameter selection.

Cellular and Tissue Responses to Electric Fields

Beyond membrane permeabilization, electroporation elicits:

- Cytoskeletal Disruption: Transient depolymerization of actin filaments, facilitating macromolecular mobility.
- Vascular Effects: Transient vasoconstriction followed by increased vascular permeability ("vascular lock"), potentially enhancing extravasation and retention of therapeutic complexes.
- Inflammatory Signaling: Danger-associated molecular pattern (DAMP) release, including ATP and HMGB1, contributing to immunogenic cell death and antitumor immunity.

Reversible versus Irreversible Electroporation

- Reversible Electroporation: Goal of nucleic acid delivery. Cell viability preserved; membrane reseals within minutes. Requires precise parameter selection to avoid transition to irreversible damage.
- Irreversible Electroporation: Fields exceeding reversible threshold induce permanent membrane destabilization and cell death. Clinically approved for tumor ablation (NanoKnife). In the proposed platform, irreversible electroporation is undesirable except as potential adjunctive cytoreductive component.

Electrotransfer Mechanisms for Macromolecular Complexes

The transport of OMV-particle complexes during electroporation involves:

- Electrophoresis: Charged complexes migrate along electric field vector. Negatively charged nucleic acids and OMVs experience force toward anode; net

charge of complexes determines electrophoretic mobility.

- Electroosmosis: Bulk fluid flow counteracting electrophoresis in confined interstitial spaces.
- Diffusion: Post-pulse, concentration-driven transport through residual pores.

Optimal electrotransfer requires coordination between pulse parameters and complex physicochemical properties.

Delivery Protocol and Procedural Workflow

Pre-Administration Preparation of OMV-Particle Formulations

- Thawing and Dilution: Cryopreserved OMV-particle complexes are thawed rapidly at 37° C, diluted to working concentration in isotonic, low-conductivity buffer (e.g., 10% sucrose, 5% glycerol, 10 mM histidine). High conductivity buffers induce excessive heating during electroporation.
- Viability Assessment: Post-thaw particle size, encapsulation efficiency, and OMV integrity confirmed prior to administration.
- Dose Determination: Based on tumor volume, typically 50-200 μg nucleic acid per cm³ tumor, with OMV concentration optimized to deliver particle:OMV ratios determined in preclinical optimization.

Intratumoral and Peritumoral Injection Strategies

- Direct Intratumoral Injection: Single or multi-site injection using 25-30 gauge needles. Fanning technique distributes formulation throughout tumor volume. Ultrasound or CT guidance may facilitate accurate placement in non-palpable tumors.
- Peritumoral Injection: Complementary administration into tumor margin, targeting invasive front and draining lymphatics.
- Volume Considerations: Injection volume should not exceed approximately 30% of tumor volume to avoid excessive interstitial pressure and leakage.

Electroporation Device Selection and Parameter Optimization

- Clinical Electroporators: Cliniporator (IGEA), OncoSec TDS, BTX ECM 830. Selection criteria include pulse parameter range, electrode compatibility, impedance monitoring, and regulatory status.

- Parameter Optimization: Field strength empirically determined from tumor type and size. Initiating parameters: 600-1000 V/cm, 100 μ s pulses, 8 pulses, 5 Hz. Real-time current monitoring detects excessive conductivity changes indicating irreversible damage.

- Electrode Insertion: Needle electrodes placed to bracket injection sites, typically 4-10 mm spacing. Parallel alignment ensures field homogeneity.

Real-Time Monitoring and Impedance Feedback

Modern electroporators measure tissue impedance throughout pulse delivery. Impedance decreases as membrane permeabilization occurs. Abrupt impedance rises may indicate arcing or electrode-tissue interface failure. Impedance feedback enables adaptive pulse delivery, terminating stimulation when target permeabilization is achieved.

Post-Electroporation Care and Safety Considerations

- Immediate Post-Procedure: Brief application of pressure at needle sites to minimize bleeding/hematoma. Analgesia as indicated for procedure-related discomfort.

- Monitoring: Vital signs, tumor site assessment, and systemic inflammatory response monitoring for 2-4 hours post-procedure.

- Repeat Administration: Platform amenable to repeated cycles at intervals determined by transgene expression kinetics (typically 1-4 weeks).

Standard Operating Procedure Documentation

Comprehensive SOPs must address:

1. Formulation preparation and quality release
2. Tumor volume measurement and electrode selection
3. Injection technique and volume distribution
4. Electrode placement and verification

5. Pulse parameter programming and delivery
6. Adverse event management
7. Post-procedure sample collection for correlative studies

Intracellular Trafficking and Cargo Release

Mechanisms of OMV-Mediated Cytosolic Entry

OMVs internalized by tumor cells primarily via:

- Macropinocytosis: Actin-driven membrane ruffling engulfing large volumes of extracellular fluid. Favored by many cancer cells as a nutrient-scavenging mechanism.

- Lipid Raft-Mediated Endocytosis: Exploitation of OMV membrane composition rich in cholesterol and sphingolipids, interacting with raft-associated receptors.

- Membrane Fusion: Direct fusion of OMV lipid bilayer with plasma membrane, depositing luminal contents into cytoplasm. Fusion efficiency correlates with membrane fluidity and may be enhanced by electroporation-induced membrane destabilization.

Endosomal Escape Enhancement via Electroporation

Endosomal entrapment represents the primary barrier to nucleic acid delivery. Electroporation facilitates escape through:

- Physical Disruption: Electric fields penetrate endosomal membranes, creating pores for cargo release.

- Electro-endosomal Lysis: Osmotic imbalance and localized heating contribute to endosome rupture.

- Calcium Flux: Electroporation-mediated calcium influx triggers endosomal destabilization via calmodulin-dependent pathways.

Fate of Nucleic Acid Cargo in the Cytoplasm

- mRNA: Immediately available for ribosome engagement. Translation efficiency influenced by 5' UTR structure, codon optimization, and cytoplasmic nucleases.

- saRNA: Replication complex formation required; initial translation produces replicase complex, which amplifies full-length and subgenomic RNA species.

- DNA: Requires nuclear translocation. Cytoplasmic DNA sensing (cGAS-STING) can induce type I interferon responses, contributing to antitumor immunity but potentially limiting transgene expression duration.

Nuclear Translocation Requirements for DNA-Based Systems

Quiescent tumor cells (common in solid tumors) maintain intact nuclear envelopes. DNA entry occurs only during mitosis when nuclear envelope disassembles, or via active import using nuclear localization signal (NLS)-tagged DNA-binding proteins. Electroporation does not directly permeabilize nuclear membranes at conventional parameters. Thus, DNA-based cargo exhibits delayed expression kinetics compared to RNA.

Protein Translation and Post-Translational Processing

- Antibody Assembly: Full-length IgG requires coordinated translation of heavy and light chains, endoplasmic reticulum (ER) translocation, disulfide bond formation, and glycosylation. Tumor cells generally support these processes, though productivity varies by cell type.

- scFv and BiTEs: Single polypeptide chains, no assembly requirement; secretion more efficient than full-length antibodies.

- Quality Control: ER chaperones and unfolded protein response (UPR) may limit production of complex proteins in non-B-cell lineages.

Antibody Secretion Pathways and Extracellular Distribution

- Constitutive Secretion: Default pathway for antibodies lacking ER retention signals. Rate determined by translation efficiency and secretory pathway capacity.

- Local Distribution: Secreted antibodies diffuse through interstitial space, establishing concentration gradient from production source. Binding to

extracellular matrix components may restrict diffusion or create local depot.

- Lymphatic Drainage: Antibodies entering tumor lymphatics reach tumor-draining lymph nodes, potentially modulating nodal immune responses.

In Situ Antibody Production and Therapeutic Action Kinetics of Transgene Expression Following Electrotransfer

- mRNA: Expression detectable within 2-4 hours, peak at 24-48 hours, declining to baseline by 7-10 days.

- saRNA: Delayed peak (48-72 hours) but prolonged expression (2-4 weeks) due to intracellular amplification.

- DNA: Onset 24-48 hours (requiring nuclear import), peak 3-7 days, duration weeks to months depending on episomal retention and promoter activity.

Tumor Cell Biofactory Concept and Sustainability

Transfected tumor cells function as localized bioreactors, continuously producing and secreting therapeutic antibodies. Advantages over systemic protein administration include:

- Sustained Exposure: Continuous antibody presence at tumor site versus peak-trough fluctuations with bolus protein injection.

- Reduced Systemic Exposure: Local production minimizes serum concentrations, reducing immune-related adverse events.

- Cost Efficiency: One-time nucleic acid synthesis versus repeated protein manufacturing and purification.

Antibody Specificity and Functional Activity

- Target Binding: Confirmed by ELISA, surface plasmon resonance, or flow cytometry using tumor cell lines expressing target antigen.

- Effector Function: Fc-mediated activities (ADCC, CDC) require appropriate IgG subclass and glycosylation. Tumor-produced antibodies exhibit

variable glycosylation patterns compared to CHO-produced biologics.

- Functional Assays: T-cell activation assays for checkpoint inhibitors; cytotoxicity assays for tumor-targeting antibodies.

Paracrine and Autocrine Signaling within the TME

- Autocrine: Antibody binding to antigen on producing cell surface; relevant for tumor-targeting antibodies inducing apoptosis or growth arrest.

- Paracrine: Diffusion to neighboring cells; dominant mode for checkpoint inhibitors and immunostimulatory antibodies, which act on tumor-infiltrating lymphocytes and antigen-presenting cells.

- Bystander Effects: Soluble factors induced by antibody activity (e.g., $\text{IFN } \gamma$ from activated T cells) propagate antitumor effects beyond antibody diffusion radius.

Immune Cell Recruitment and Activation

- Checkpoint Inhibition: PD-1/PD-L1 blockade reactivates exhausted tumor-infiltrating lymphocytes (TILs), restoring cytotoxic function.

- T-cell Engagement: BiTEs recruit and activate CD8+ T cells regardless of TCR specificity, overcoming MHC downregulation.

- Antigen Presentation: Immunostimulatory antibody-induced DC maturation enhances cross-presentation of tumor antigens, priming novel T-cell specificities (epitope spreading).

- OMV Contribution: Desharpened OMVs retain TLR4 agonist activity sufficient for DC maturation, providing adjuvant effect complementing antibody-mediated immune activation.

Synergistic Effects with OMV-Mediated Danger Signals

OMV components (detoxified LPS, lipoproteins) engage TLR4 and TLR2 on innate immune cells within TME, inducing:

- Inflammatory Cytokines: $\text{TNF } \alpha$, IL-12, type I interferons promoting Th1 polarization.

- Neutrophil Recruitment: Early innate response facilitating antigen uptake and presentation.

- Inflammasome Activation: Certain OMV preparations activate NLRP3 inflammasome, releasing $\text{IL-1 } \beta$ and IL-18.

The combination of OMV adjuvant activity and antibody-mediated immune modulation creates an immunostimulatory TME permissive for sustained antitumor immunity.

Preclinical Evidence and Proof-of-Concept Studies

In Vitro Models: OMV Uptake and Transfection Efficiency

- Cell Lines: Murine (4T1, B16-F10, CT26) and human (MDA-MB-231, A375, HCT116) cancer cell lines representing diverse histologies.

- Uptake Quantification: Fluorescently labeled OMVs (DiD, DiR membrane dyes) and nanoparticles (Cy5-RNA) enable flow cytometric and confocal assessment of association efficiency, kinetics, and intracellular localization.

- Transfection Efficiency: Encoded reporter genes (GFP, luciferase, secreted embryonic alkaline phosphatase) quantify functional delivery and expression kinetics.

- Optimization Studies: Systematic variation of particle-to-OMV ratio, electroporation parameters, and formulation buffers to maximize transfection while minimizing cytotoxicity.

Ex Vivo Tumor Slice Culture Systems

- Methodology: Fresh human or murine tumor specimens sectioned (200-300 μm) using vibratome, maintained at air-liquid interface, treated with OMV-particle complexes \pm electroporation.

- Advantages: Preserved TME architecture, stromal components, and immune infiltrate; enables evaluation of platform performance in patient-derived tissues; reduces animal use.

- Readouts: Transfection efficiency (cell-type specific via immunohistochemistry/immunofluorescence), cytokine secretion, viability.

Subcutaneous and Orthotopic Tumor Xenograft Models

- Subcutaneous Models: Human tumor cell lines implanted in immunodeficient mice (athymic nude, NSG). Enable human-specific antibody evaluation; lack adaptive immune compartment limiting assessment of immunotherapeutic mechanisms.

- Orthotopic Models: Tumor implantation in anatomically relevant sites (e.g., mammary fat pad for breast cancer, intracranial for glioblastoma). Better recapitulate TME, metastasis patterns; technically challenging for electroporation access.

- Dosing Regimens: Single versus multi-dose schedules; dose-ranging studies to establish minimal effective dose.

Syngeneic Murine Models for Immunocompetent Evaluation

- Models: CT26 (colon), 4T1 (breast), B16-F10 (melanoma), MC38 (colon) in immunocompetent BALB/c or C57BL/6 mice.

- Advantages: Intact immune system enables assessment of checkpoint inhibitor encoding, BiTE activity, and OMV adjuvant effects; facilitates tumor rechallenge studies to evaluate immunological memory.

- Limitations: Species specificity: human antibody sequences may be immunogenic in mice; murine cross-reactive or fully murine antibodies required.

Biodistribution, Pharmacokinetics, and Toxicity Profiling

- Biodistribution: Radiolabeled (^{111}In , ^{64}Cu) or near-infrared fluorescently labeled OMVs and nanoparticles tracked by SPECT/PET/CT or IVIS imaging. Quantification of tumor accumulation, retention, and clearance organs (liver, spleen, kidneys).

- Transgene Biodistribution: Bioluminescence imaging (luciferase reporter) or tissue qRT-PCR for vector RNA/DNA to assess tumor-selective expression versus off-target transfection (particularly following electroporation of adjacent normal tissue).

- Toxicity: Acute (24-72 hours): clinical observations, serum chemistry, hematology, cytokine panels (IL-6, TNF α , IFN γ). Subacute/chronic (14-28 days): histopathology of injection site, draining lymph nodes, major organs.

Efficacy Endpoints: Tumor Growth Inhibition and Survival

- Primary Endpoints: Tumor volume measurement (caliper, ultrasound, MRI), time to progression, overall survival.

- Secondary Endpoints: Intratumoral antibody quantification (ELISA from tumor lysates), pharmacodynamic markers (target engagement, downstream signaling), immune infiltrate characterization (flow cytometry, multiplex IHC).

- Rechallenge Studies: In syngeneic models, cure of primary tumor followed by contralateral tumor implantation assesses immunological memory and systemic antitumor immunity.

Safety, Immunogenicity, and Biocompatibility

Desharpened OMV Residual Toxicity Assessment

Quantitative assays define the therapeutic window for engineered OMVs:

- TLR4 Reporter Assays: HEK-Blue or similar cell lines expressing TLR4 and NF- κ B-inducible SEAP; EC50 comparison of desharpened versus native OMVs guides acceptable attenuation threshold (typically 100-1000 fold reduction).

- Human PBMC Cytokine Release: Multiplex quantification of IL-6, TNF α , IL-1 β , IL-12p70 following OMV stimulation. Pro-inflammatory cytokine induction should be reduced but not ablated, as residual adjuvanticity is desirable.

- Local Reactogenicity: Intradermal injection in rabbit models with histopathological assessment of injection site inflammation.

Anti-Vector Immune Responses and Neutralization Risks

Repeated OMV administration may induce adaptive immunity against bacterial components:

- Anti-OMV Antibodies: ELISA quantification of IgG against OMV lysate. Neutralizing activity assessed by inhibition of OMV uptake by macrophages or tumor cells in vitro.

- T-cell Responses: ELISpot for IFN γ -secreting T cells following OMV restimulation.

- Impact on Repeat Dosing: Pre-existing immunity may accelerate clearance and reduce transfection efficiency; strategies include serotype switching (OMVs from different bacterial species), PEGylation, or dose escalation.

Anti-Drug Antibodies against Encoded Therapeutics

Tumor-produced antibodies are human proteins; however, glycosylation differences and high local concentrations may rarely elicit anti-drug antibodies (ADA). Assessment:

- ADA Assays: Bridging ELISA or electrochemiluminescence immunoassays using purified recombinant antibody identical to encoded sequence.

- Neutralizing ADA: Cell-based assays measuring inhibition of antibody-antigen binding or functional activity.

Genomic Integration Risk for DNA-Based Cargo

Plasmid and minicircle DNA remain predominantly episomal; however, rare random integration events cannot be excluded. Risk assessment:

- Integration Frequency: Linear amplification-mediated (LAM)-PCR or next-generation sequencing of genomic DNA from transfected cells following extended culture.

- Tumor Suppressor Locus Integrity: In silico analysis of plasmid sequences for homology to human genome; avoid inclusion of sequences promoting recombination.

- Regulatory Perspective: Integration frequency below spontaneous mutation rate generally considered acceptable for non-germline therapeutics.

Off-Target Electroporation Effects and Tissue Damage

Electroporation confined to tumor volume minimizes off-target effects; however:

- Muscle Stimulation: High-frequency pulses (>1 kHz) reduce neuromuscular stimulation; electrode placement avoids major nerve trunks.

- Thermal Damage: Joule heating quantified by temperature probes; limited by low-conductivity buffers, adequate inter-pulse intervals (<1° C rise typically acceptable).

- Fibrosis and Scarring: Chronic electroporation may induce fibrotic changes; mitigated by minimizing treatment frequency and field strength.

Regulatory Considerations for Combination Products

The platform constitutes a combination product (biological/device) with complex regulatory pathway:

- FDA: Likely Center for Biologics Evaluation and Research (CBER) lead, with Office of Combination Products coordination. Device component (electroporator) reviewed separately or as part of combination application.

- EMA: Committee for Advanced Therapies (CAT) responsible for gene therapy medicinal products; electroporation device reviewed under Medical Device Regulation.

- Key Considerations: Chemistry, manufacturing, and controls (CMC) requirements for OMV-based biologicals; demonstration of device safety and performance in combination; post-marketing surveillance for rare adverse events.

Translational Pathway and Clinical Development

Manufacturing Scale-Up and GMP Compliance

- OMV Production: Transfer from research-scale shake flasks to GMP-compliant stirred-tank bioreactors with single-use technology. Process validation includes consistency across three consecutive batches.

- Nucleic Acid Manufacturing: Synthetic mRNA produced by in vitro transcription using GMP-grade enzymes and nucleotides; plasmid DNA fermentation

in E. coli K-12 strains; final product purified by oligo-dT affinity (mRNA) or anion exchange (DNA).

- LNP Formulation: Microfluidic mixing technology scaled from bench-top (NanoAssembly) to GMP production systems (Precision Nanosystems, Cytiva).

- Formulation and Fill-Finish: Aseptic filling into single-dose vials; lyophilization development for thermal stability.

Formulation Stability and Lyophilization Strategies

- Liquid Formulation: OMV-particle complexes stable for 2-8 hours at 2-8 ° C; frozen formulations (-80 ° C) stable for ≥ 6 months. Frozen supply chain established for mRNA vaccines.

- Lyophilization: Cryoprotectant optimization (trehalose, sucrose) preserves particle size and encapsulation efficiency through freeze-drying. Residual moisture <2% essential for stability.

- Accelerated Stability Studies: 25 ° C/60% RH and 40 ° C/75% RH time points inform real-time stability predictions and provisional shelf-life assignments.

Electroporation Device Adaptation for Human Use

- Clinical Electrode Development: Endoscopic, laparoscopic, or percutaneous electrode configurations required for deep-seated tumors (lung, liver, pancreas). Collaboration with device manufacturers for custom electrode arrays.

- Treatment Planning Software: Finite element modeling interface importing patient CT/MRI to predict field distribution and guide electrode placement. Integration with electroporator software.

- Human Factors Engineering: User interface optimization for oncology clinicians; training programs for procedural standardization.

First-in-Human Trial Design Considerations

- Population: Patients with advanced, treatment-refractory solid tumors; accessible lesions (cutaneous, subcutaneous, nodal) for initial safety evaluation.

- Design: 3+3 or BOIN dose escalation (nucleic acid dose, OMV dose, electroporation parameters)

with expansion cohort at recommended phase 2 dose (RP2D).

- Endpoints:

- Primary: Safety (DLT rate, adverse event profile)

- Secondary: Transgene expression in tumor biopsies (RNAscope, IHC), objective response rate (RECIST v1.1)

- Exploratory: Immune correlates (TIL phenotyping, TCR sequencing)

Patient Selection and Tumor Accessibility Criteria

- Inclusion: Measurable disease amenable to intratumoral injection and electroporation; ECOG 0-1; adequate organ function.

- Exclusion: Coagulopathy; cardiac pacemaker/defibrillator (electroporation contraindication); active autoimmune disease requiring systemic immunosuppression.

- Tumor Types: Prioritize tumors with established electroporation experience (melanoma, head and neck squamous cell carcinoma, breast cancer cutaneous metastases).

Biomarkers for Response Monitoring

- Pharmacodynamic: Pre- and post-treatment tumor biopsies: quantification of encoded antibody by mass spectrometry or immunohistochemistry; assessment of target engagement (e.g., PD-L1 occupancy); downstream signaling inhibition.

- Predictive: Baseline tumor immune phenotype (inflamed, excluded, desert) may predict responsiveness to locally produced checkpoint inhibitors.

- Surrogate: Circulating tumor DNA (ctDNA) dynamics as early indicator of tumor cell killing.

Comparative Analysis with Alternative Platforms

Viral Vectors: Advantages and Limitations

- Adenovirus (AdV): High transduction efficiency; broad tropism; episomal maintenance. Limitations: pre-existing immunity; hepatotropism; immunogenicity limiting repeat dosing.

- Adeno-Associated Virus (AAV): Excellent safety profile; sustained expression. Limitations: small packaging capacity (<4.7 kb) precluding full-length antibody genes; pre-existing neutralizing antibodies; nuclear requirement.

- Lentivirus (LV): Stable integration; transduces dividing and non-dividing cells. Limitations: integration-associated genotoxicity risk; manufacturing complexity; cost.

- OMV-Electroporation Contrast: Non-viral avoids pre-existing anti-vector immunity; manufacturing scalability; no genome integration; comparable or superior transfection efficiency with optimized electroporation.

Lipid Nanoparticles and COVID-19 Legacy Technologies

- Systemic LNP-mRNA: Clinically validated; rapid expression; repeat dosing feasible. Limitations: hepatic tropism after IV administration; poor penetration into solid tumors; inflammatory toxicities at high doses.

- Intratumoral LNP-mRNA: Direct injection obviates targeting requirement; LNP retention at injection site variable; electroporation enhances uptake 10-100 fold versus LNP alone.

- OMV-Electroporation Contrast: OMVs provide fusogenic and immunostimulatory functions absent from synthetic LNPs; electroporation synergizes with OMV properties for enhanced transfection.

Polymeric Nanoparticles and Dendrimers

- PLGA Nanoparticles: Sustained release; excellent stability. Limitations: lower transfection efficiency than lipids; organic solvent residues.

- Dendrimers: Defined molecular structure; multivalent surface modification. Limitations: cytotoxicity at high generations; limited clinical experience.

Cell-Based Delivery Systems

- CAR-T Cells: Potent antitumor activity in hematologic malignancies. Limitations: solid tumor efficacy limited; manufacturing complexity; cytokine release syndrome.

- Tumor-Infiltrating Lymphocytes (TIL): Polyclonal antitumor activity. Limitations: ex vivo expansion challenges; variable efficacy.

- Neutrophil/Macrophage Carriers: Exploit tumor-homing properties. Limitations: cargo loading constraints; donor variability.

Direct Electrotransfer of Naked Nucleic Acids

- Established Precedent: DNA electrotransfer extensively studied; safe and effective for vaccination and local therapy. Limitations: unprotected DNA susceptible to degradation; requires high doses; OMV-mediated protection reduces required dose by 10-100 fold.

Positioning of OMV-Electroporation Hybrid Platform

- Unique Value Proposition: Combines carrier-mediated protection and cell interaction (OMVs) with physical driving force overcoming membrane barriers (electroporation). Neither component alone achieves comparable efficacy. Platform versatility permits cargo switching without carrier redesign.

- Ideal Indications: Accessible solid tumors with immunosuppressive TMEs; combination therapy with systemic checkpoint blockade; neoadjuvant therapy for resectable disease.

Future Directions and Emerging Innovations

Programmable OMVs with Synthetic Biology Circuits

- Engineered Production Strains: Incorporation of quorum sensing circuits to synchronize OMV release at high cell density; lysis cassettes for induced vesiculation.

- Surface Display: Fusion of targeting ligands (scFv, nanobodies, DARPins) to OMV surface proteins (ClyA, OmpA, OprI) for tumor-selective binding.

- Cargo Loading: Directed packaging of heterologous proteins into OMV lumen using periplasmic targeting signals.

Multi-Antigen and Combination Antibody Encoding

- Polycistronic Vectors: 2A peptide-linked expression of multiple antibody specificities from single mRNA.

- Multiplexed LNP: Encapsulation of distinct mRNA species within single nanoparticle; independent titration of each component.

- Antibody-Cytokine Fusions: Simultaneous checkpoint blockade and local cytokine therapy.

CRISPR-Cas Delivery for Tumor Gene Editing

- Transient Cas9 Expression: mRNA encoding Cas9 nuclease with chemically modified sgRNA; permanent disruption of immunosuppressive genes (PD-L1, TGF β R2) or oncogenic drivers.

- Base Editing and Prime Editing: Precision gene correction without double-strand breaks; reduced off-target risk.

Microbubble-Enhanced Sonoporation as Alternative

- Ultrasound-Mediated Delivery: Cavitating microbubbles transiently permeabilize cell membranes; non-invasive; applicable to deep tissues; lower efficiency than electroporation currently; combination with OMV-microbubble hybrids under investigation.

Closed-Loop Electroporation with Imaging Guidance

- MRI-Guided Electroporation: Interventional MRI enables real-time electrode placement verification and field distribution visualization; MR-compatible electrodes under development.

- Impedance Tomography: Reconstruction of tissue conductivity changes during electroporation to verify permeabilization extent.

Artificial Intelligence for Parameter Optimization

- Machine Learning Models: Training datasets from in vitro and in vivo electroporation experiments; prediction of optimal pulse parameters for tumor types based on electrical properties, size, and location.

- Automated Parameter Selection: Integration into clinical electroporators; user inputs tumor characteristics; device outputs recommended settings.

Technical Challenges and Mitigation Strategies

Complex Manufacturing and Supply Chain Integration

- Challenge: Three distinct manufacturing processes (OMV production, nucleic acid synthesis, nanoparticle formulation) must be coordinated and integrated.

- Mitigation: Develop modular manufacturing with stable intermediates; define specifications at each integration step; establish partnerships with specialized contract development and manufacturing organizations (CDMOs).

Batch-to-Batch Variability in OMV Production

- Challenge: Bacterial culture inherently variable; OMV yield, protein composition, and LPS structure may fluctuate.

- Mitigation: Implement quality by design (QbD) principles; define critical process parameters (CPPs) and critical quality attributes (CQAs); develop in-process controls; establish reference standards.

Nucleic Acid Instability and Cold Chain Requirements

- Challenge: mRNA and LNP-mRNA require frozen storage; vulnerability to thermal excursion.

- Mitigation: Lyophilization development; formulation optimization for refrigerated stability; cold chain monitoring technologies; explore self-amplifying RNA for improved thermal stability.

Tumor Heterogeneity and Variable Transfection Rates

- Challenge: Not all tumor cells within heterogeneous population equally permissive to transfection; stromal cells (cancer-associated fibroblasts, endothelial cells) also transfected.

- Mitigation: Stromal transfection may contribute therapeutically (e.g., antibody production by CAFs); combinatorial targeting of multiple cell populations; enhance perfusion and distribution with hyaluronidase co-administration.

Electroporation Accessibility in Deep-Seated Tumors

- Challenge: Lung, liver, pancreatic tumors not amenable to percutaneous electrode insertion without traversing critical structures.

- Mitigation: Endobronchial electrodes for lung tumors; endoscopic ultrasound-guided electrodes for pancreatic tumors; transarterial electroporation using catheter-based electrodes; hybrid approach with irreversible electroporation ablation.

Cost-Effectiveness and Reimbursement Projections

- Challenge: Advanced therapy manufacturing costs substantial; electroporation procedure reimbursed under existing codes for electrochemotherapy but platform-specific codes lacking.

- Mitigation: Health economic modeling demonstrating cost-effectiveness versus systemic biologics (e.g., pembrolizumab at \$100,000+ per year); engage payers early; develop value-based pricing strategies.

Conclusion and Strategic Outlook

Summary of Platform Advantages

The OMV-electroporation hybrid platform represents a significant advance in locoregional cancer therapy, offering:

1. Synergistic Delivery: Electroporation and OMVs cooperatively overcome the two principal barriers to nucleic acid therapeutics---membrane permeability and endosomal entrapment---achieving transfection efficiencies unattainable by either modality alone.

2. Versatility: Platform readily adapts to diverse antibody formats and other protein therapeutics; OMV carrier functions independently of specific cargo sequence.

3. Safety: Desharpended OMVs exhibit substantially reduced systemic toxicity while retaining desirable adjuvanticity; electroporation confined to target tissue minimizes off-target effects.

4. Immunogenicity Profile: Non-viral platform avoids pre-existing anti-vector immunity limiting viral vectors; repeat administration feasible.

5. Manufacturing Scalability: Bacterial OMV production and synthetic nucleic acid manufacturing established at industrial scale; trajectory toward affordable, widely accessible therapy.

Remaining Knowledge Gaps

Critical questions warranting further investigation:

- Optimal desharpening balance: how much TLR4 agonism must be retained to maximize therapeutic index?

- Long-term fate of OMVs in vivo: degradation kinetics, persistence of bacterial components.

- Durability of antitumor immunity following platform-mediated immunotherapy: memory responses, protection against recurrence.

- Activity in immunologically "cold" tumors: can OMV adjuvanticity plus local checkpoint inhibition sufficiently inflame TME?

Roadmap for Clinical Translation

- *2024-2025*: Preclinical optimization and IND-enabling studies (GLP toxicology, biodistribution); device adaptation for human use; regulatory engagement for combination product classification.

- *2025-2027*: First-in-human phase 1 trial; establish safety and RP2D; preliminary efficacy signals.

- *2027-2029*: Randomized phase 2 trials in selected indications; biomarker validation; phase 3 planning.

- *2030+*: Potential regulatory approvals; post-marketing studies; platform expansion to additional cancer types and non-oncology applications.

Potential Beyond Oncology: Infectious Diseases and Genetic Disorders

- Infectious Diseases: Intradermal electroporation of OMV-mRNA encoding broadly neutralizing antibodies for HIV-1 pre-exposure prophylaxis; intranasal delivery for respiratory pathogens.

- Genetic Disorders: Localized production of missing enzymes or structural proteins; promoter engineering for tissue-specific expression.

- Vaccination: OMV adjuvanticity enhances immunogenicity of encoded antigens; electroporation

augments antigen expression; synergistic for T-cell priming.

Final Remarks

The convergence of bacterial vesicle engineering, synthetic nucleic acid technologies, and physical delivery methods exemplifies the power of interdisciplinary approaches to therapeutic challenges. As each component technology continues to advance, the combinatorial platform will evolve, offering increasingly sophisticated and effective interventions. The work described in this document represents not an endpoint but a foundation---a framework upon which future innovations in targeted, local, sustainable protein therapeutics will be built. The journey from laboratory concept to clinical reality is arduous, demanding rigorous science, careful engineering, and steadfast commitment. Yet the potential reward---a versatile, potent, and accessible platform capable of transforming cancer therapy and beyond---justifies the endeavor.

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