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Isolating T-Cell Potent Lentiviral Vector Subpopulations through Rational Design of Nanofiber Anion-Exchange Adsorbents

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ABSTRACT

Generation of CAR-T cell therapies relies predominantly on lentiviral vectors (LVs). As in vivo CAR-T trials progress, demand for larger quantities of higher-potency LV is expected. However, primary capture by anion-exchange chromatography (AIEC) remains a major manufacturing bottleneck from use of adsorbents not tailored to LVs, leading to complex binding phenomena such as time-dependent product loss and a heterogeneous two-peak elution profile arising from two distinct LV subpopulations (Peak 1 and Peak 2). Current industry practice is to collect both peaks to maximize total product recovery. Yet peak-specific differences in product potency and demand for higher purity products for use in in vivo CAR-T cell therapy will likely drive a need to separate these LV species. Electrospun nanofibers offer a promising next-generation adsorbent for addressing these challenges, making it essential to understand how Q-nanofiber design affects LV recovery and Peak 1/Peak 2 selectivity. This work indicates that Peak 1 and Peak 2 LVs differ in their T-cell transduction potency. Despite generating higher numbers of CD3⁺GFP⁺ T cells at low doses, Peak 1 sees reducing T-cell viability and %CD3⁺GFP⁺ cells at higher doses, likely from an inhibitory effect of VSV-G vesicles that are co-eluted with Peak 1 LV during AIEC. In contrast, Peak 2 LV maintained increasing %CD3⁺GFP⁺ T cells and high viability across doses. A Q-nanofiber adsorbent enabling high-recovery AIEC while increasing LV peak separation was therefore required to isolate the more potent Peak 2 LV subpopulation. Increasing nanofiber porosity (0.4–0.6 v/v) enhanced LV recovery by reducing time-dependent loss, whereas Peak Separation increased with ligand density and nanofiber diameter. An optimal Q-nanofiber structure was therefore identified and validated across two LV batches, achieving high recovery (64%–82%) while isolating Peak 2 LV from the major VSV-G elution.

1 | Introduction

The Cell and Gene Therapy (C>) industry has rapidly expanded since Glybera's approval in 2012 (Ylä-Herttuala 2012), with 1894 clinical trials, \$11.7 billion in global investment and eight breakthrough approvals in 2023 alone (Alliance for Regenerative Medicine 2024). This growth is driving increased

demand for clinical-grade viral vectors, used for gene delivery. Lentiviral vectors (LVs) are preferred for *ex vivo* modification of patient cells due to their ability to stably deliver up to ~10 kb payloads, irrespective of mitotic state (Lewis et al. 1992; Mátrai et al. 2010; Naldini et al. 1979). Because of this, LVs are the most commonly employed vector for engineering chimeric antigen receptor (CAR)-T cell therapies for treatment of various

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cancers, and there are now several FDA approved LV-based cell-therapy products (FDA 2025). This has made LVs a major component in C> clinical trials, accounting for 33% of known U.K. trials (Cell and Gene Therapy Catapult 2023). However, inefficient manufacturing processes lead to high costs and limited availability of LV products, creating significant supply challenges for the C> industry. Furthermore, the first cohort of in vivo CAR-T therapies are now entering clinical trials (Bot et al. 2025). As such, LV products are transitioning from a raw material in *ex vivo* cell therapy manufacture, to a final injectable drug product for in vivo CAR-T cell engineering (Mullard 2024). This shift places even greater emphasis on the manufacturability and quality of LV products. Effective large-scale purification, typically achieving only 10%–25% product recovery with poor separation performance, remains the main obstacle to cost-effective, robust manufacturing (Comisel et al. 2021; Ansorge et al. 2010; Valkama et al. 2020).

Anion-exchange chromatography (AIEX) is the predominant technique used for primary capture of LV's. While high recoveries using newer commercial AIEX technologies are now reported, most applications still typically yield only 30%–40% functional product, constituting most of the downstream process losses (Valkama et al. 2020; Moreira et al. 2021; Pamenter et al. 2025). These poor recoveries stem from the use of adsorbent materials not designed for LV AIEX, and a lack of understanding of the complex adsorption phenomena that stem from LVs variable and intricate structure. Most clinical LVs are 3rd generation and derived from Human Immunodeficiency Virus Type 1 (HIV-1), a spherical, single-stranded RNA virus, 80–120 nm in size, with a p24 protein core and a lipid bilayer embedded with proteins from the production cell membrane (King 1994; Nguyen and K Hildreth 2000). Our previous work identified a key mechanism responsible for low AIEX recoveries, where time-spent in the adsorbed state increased LV product loss (Pamenter et al. 2023). This was attributed to a conformational spreading that caused irreversible binding from strong multipoint attachment with AIEX ligands. These effects were mitigated when using adsorbents with lower LV-adsorbent interaction strengths (Pamenter et al. 2025).

Linear gradient elution of LVs typically results in broad, heterogeneous elution profiles, with LVs eluting across a wide range of NaCl concentrations (150–1500 mM) (Pamenter et al. 2023, 2024; Ghosh et al. 2022; Yamada et al. 2003). This is typically characterized by a “two-peak” elution pattern, where a low-salt (~400 mM NaCl) and a high-salt peak (~950 mM NaCl) are observed (Pamenter et al. 2023; Yamada et al. 2003). Our previous work identified that these two peaks arise from distinct LV subpopulations, each characterized by unique envelope compositions (Pamenter et al. 2024). Peak 1 (weaker binding) LVs are thought to bind to AIEX membranes via the phospholipid membrane and/or envelope proteins and thus hypothesized to have greater envelope protein density. In contrast, Peak 2 (stronger binding) LVs are thought to have envelopes enriched in highly charged glycosaminoglycans (GAGs) as they were shown to bind via these species. Crucially, these two subpopulations were shown to vary twofold in their gene-delivery efficiency in primary T cells at low multiplicity-of-infection (MOI), agreeing with similar reports in the literature (Yamada et al. 2003).

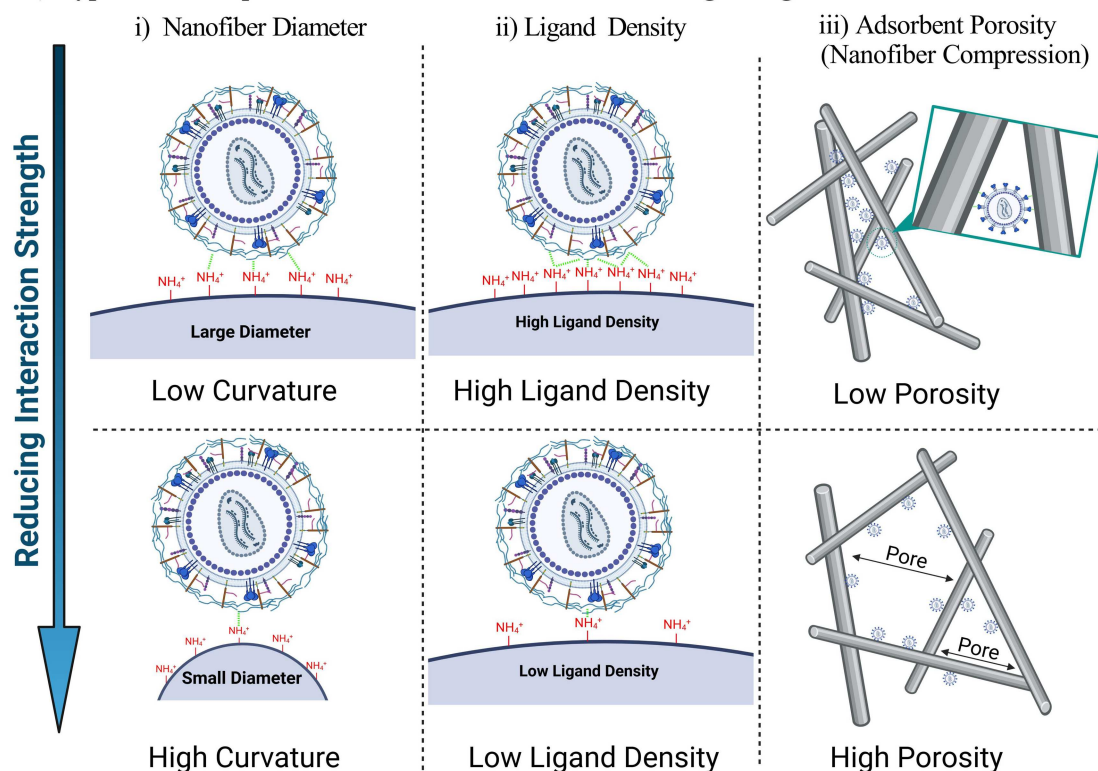
These distinct LV subpopulations present significant challenges for effective purification. High salt concentrations required to elute both LV peaks, and thus maximize recovery, can compromise vector functionality and co-elute impurities like hcDNA. This has driven developers to pursue adsorbents that elute at lower NaCl and produce tighter, superimposed peaks to maximize recovery at the lowest salt concentrations possible (Pamenter et al. 2025; Astrea Bioseparations 2025). However, these adsorbents are limited in their ability to selectively isolate potent LV variants, which may be needed if efficacy differences are shown. Furthermore, a host of product-like particles, such as extracellular vesicles, are also likely eluted alongside LV particles. Kocot et al. (2026) recently demonstrated that weakly retained impurity particles can reduce LV transduction efficiency in a concentration-dependent manner, highlighting the potential inhibitory role of vesicular impurities on LV potency and the importance of improved chromatographic selectivity (Kocot et al. 2026).

As overall process yields improve, future LV separations may face competing priorities. Should processes aim to maximize total LV recovery, or should they prioritize potency by isolating high-performing LV subpopulations through greater selectivity? Although overall process recovery has historically taken precedence, the need for more precise separation is likely to become increasingly important as in vivo LV applications expand, since particulate impurities that reduce potency or increase toxicity may no longer be tolerated. This echoes longstanding challenges in AAV manufacturing, where insufficient separation of empty and full particles has raised clinical concerns (Kishimoto and Samulski 2022; Gomis-Fons et al. 2024). Understanding how AIEX adsorbent structure influences both time-dependent product loss and the behavior of the heterogeneous “two-peak” elution profile is therefore crucial for informing the design of next-generation LV separation materials capable of achieving high-recovery and high-selectivity.

Nanofibers are a novel class of convection-driven adsorbent materials that are seeing increasing use for the purification of viral vectors, representing one of the most promising classes of adsorbents for the separation of larger therapeutic modalities such as LV (Hardick et al. 2013; Dods et al. 2015; Achleitner et al. 2025; Ruscic et al. 2019; Turnbull et al. 2019; Ovari et al. 2024). Prior work has looked at the optimization and manipulation of nanofiber manufacturing processes to achieve variable adsorbent morphologies (Ovari et al. 2024). Ovari et al. (2024) developed a manufacturing process in which both average nanofiber diameter could be tuned from 400 to 600 nm (varying the electrospinning polymer solution) and material porosity from 0.3 to 0.6 (varying lamination pressure of the resulting electrospun sheets). The static binding capacity of pDNA was shown to differ between the nine different adsorbent structures, showing the criticality of adsorbent design in binding of larger biologics. As we hypothesized that LV binding and time dependent product loss are driven by strong multi-point interactions, tuning nanofiber morphology to give different vector-adsorbent interaction strengths may allow manipulation of the LV two peak elution profile and mitigation of time-dependent loss (Pamenter et al. 2023, 2024).

Figure 1A hypothesizes three mechanisms by which adsorbent structure could influence multipoint binding, and thus

A) Hypothesised impact of nanofiber structure on LV binding strength.



B) Experimental workflow to produce LV elution profile data set.

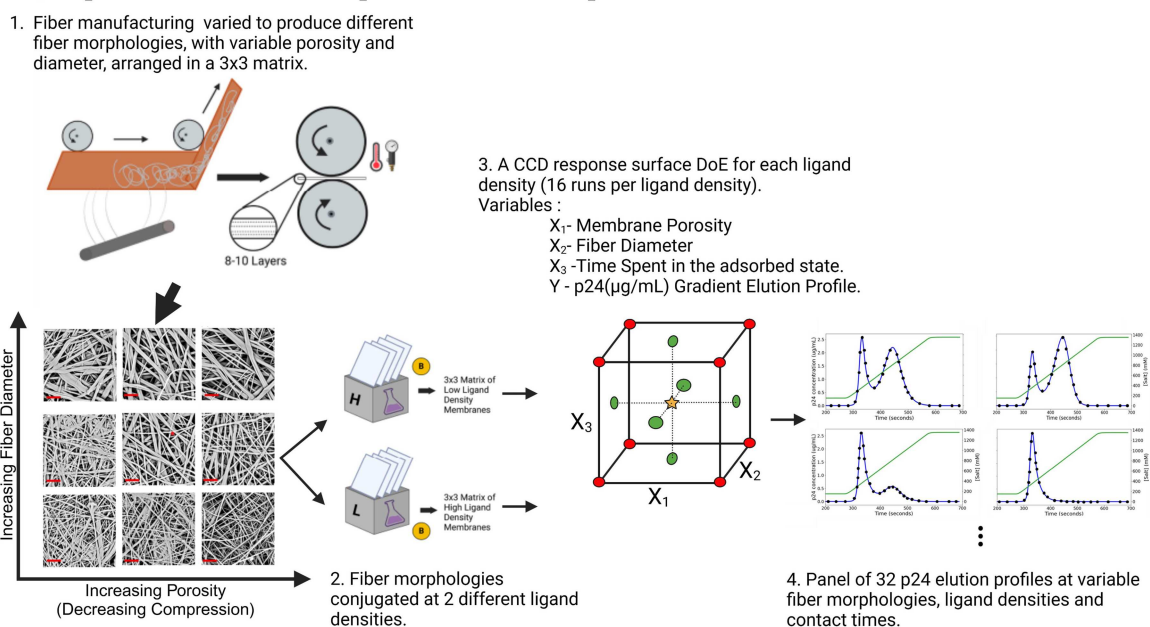


FIGURE 1 | Experimental hypothesis and methodology. (A) Schematic representation of the impact of nanofiber structural factors on multipoint interaction and the LV-adsorbent interaction strength. (B) Process flow chart explaining experimental workflow and Design of Experiment (DoE) methodology for exploring the impact of nanofibers structural factors and contact time on LV elution profiles. Nanofiber manufacture diagrams and microscopy images adapted from Ovari et al. (2024) (Ovari et al. 2024).

interaction strength. As previous studies report that RNase A stability decreases when adsorbed onto nanoparticles with larger diameters (Shang et al. 2007), we hypothesize smaller nanofiber diameters reduce LV interaction strength due to a reduced contact area associated with higher curvature (Figure 1Ai). Ligand density (Figure 1Aii) has previously been

linked to the time-dependent loss of viral vectors on nanofiber adsorbents, with charge interaction strength shown to be crucial for on-column stability in Protein and Ad5 chromatography systems (Turnbull et al. 2019; Haimer et al. 2007; Roberts and Carta 2022; Ueberbacher et al. 2010). Increased porosity (Figure 1Aiii) reduces fiber proximity and uniformity (Ovari

et al. 2024), thus lowering the presence of charged pockets and avidity effects from multiple fibers binding a single LV particle.

In this work, we elucidate which of the two LV subpopulations from the two peak profiles has higher therapeutic potency in primary T cells, before assessing how Q-nanofiber AIEX adsorbent structure can be manipulated to isolate this more efficient LV subpopulation at high recovery. To do this, we first expand our prior assessment of LV subpopulation potency (Pamenter et al. 2024) by treating primary T cells from two donors across a range of doses with purified Peak 1 and Peak 2 LV material to evaluate T-cell transduction efficiency and cytotoxicity. We then generate purified HEK293T derived particles, with and without VSV-G, from both peak 1 and peak 2 elution regions to assess for inhibitory effects of different particulate species on T-cell transduction.

These results are used to set design criteria for a new nanofiber adsorbent engineered to maximize LV recovery and subpopulation selectivity. A Design of Experiments (DoE) approach is employed to evaluate the impact of four factors (contact time, nanofiber diameter, membrane porosity, and ligand density) on LV elution behavior. Multilinear regression (MLR) is used to determine factors influencing overall LV recovery. A bimodal skewed-Gaussian statistical model is used to extract parameters describing elution profile features, then MLR is subsequently used on these parameters to quantify how nanofiber structure affects the separation of Peak 1 and Peak 2 LV and overall gradient elution profile shape. An adsorbent that achieves high recovery and selectivity is then chosen and validated for the specific purification of Peak 2 LV only.

2 | Materials and Methods

2.1 | Cell Culture & Clarification

Third-generation HIV-1 LVs, carrying a GFP transgene, were generated using the same upstream cell culture method as detailed in Pamenter et al.(2023). Two production modes, 250 mL shake flasks (SFs) (Erlenmeyer) and 7L stirred tank bioreactors (STRs) (Applikon), were employed. SFs were used to generate vesicle impurity materials. For all vesicle materials, the same cell culture method as LV was used, except viral structural plasmids (*rev*, *tat*, and *gag-pol*) were replaced with a pBluescript stuffer plasmid to maintain the same pDNA concentration as used in LV production, but with no LV particles produced. LV production in 7L STRs used identical materials as described in Pamenter et al. (2023), and the reader is referred there for a detailed description of material generation. All clarified cell culture harvest (CCH) materials were stored at -80°C prior to AIEX experiments.

2.2 | Generation and Purification of Individual AIEX Peaks and Vesicles for T-Cell Functionality Assessment

LV peak materials from different parts of the AIEX linear gradient elution were generated using the same material and methods as described in Pamenter et al.(2024). In short 1 mL Sartobind Q membranes were used to elute LV Peak 1,

Transition and Peak 2 eluates in biological triplicate. AIEX fractions were then further purified by ultrafiltration/diafiltration (UF/DF) using a 500 kDa tangential flow filter (Cytiva). All materials were processed to a final volume of 3.5 mL and filtered with 10 diavolumes of diafiltration (DF) buffer (20 mM Tris, pH 7.2, 100 mM NaCl). To generate purified vesicle preparations from Peak 1 and Peak 2, 300CV of either (+) or (−) VSV-G CCH were loaded onto the AIEX membrane at 10 CV/min. Isocratic elutions were performed at 700 and 1350 mM NaCl to produce Fractions 1 and 2. These fractions were then concentrated and diafiltered using the exact same UF/DF procedure as used for LV peaks.

2.3 | Nanofiber Adsorbent Manufacture and Device Construction

Manufacture of electrospun cellulose nanofiber adsorbents is described in detail in Ovari et al.(2024). In brief, cellulose acetate (CA) solutions at three polymer concentrations (low, medium, high) were electrospun into submicron fibers using an electrospinner (Elmarco), yielding three distinct clusters of mean fiber diameters. The resulting nonwoven mats were then laminated on a heat-and-pressure laminator (Reliant Machinery) under low, medium, and high pressures to produce adsorbents with three corresponding porosities and mean pore sizes. To chemically functionalize the different adsorbent structures, CA materials were first converted to regenerated cellulose (RC) as described in Ovari et al.(2024). RC adsorbents were then submerged in 0.5 M NaOH mixed with differing volumes of Glycidyltrimethylammonium chloride (Sigma Aldrich) to achieve the desired Q-ligand density (272 and 564 $\mu\text{mol/g}$). Single-layer sheets were stacked and laminated until the desired adsorbent mass per unit surface area was met. For each nanofiber device, a 25 mm disc was punched from the adsorbent sheet and placed into a polyether ether ketone (PEEK) sample holder. The fully assembled holder (including frits) has a hold-up volume of 1.1 mL, with the nanofiber membrane occupying ~ 0.22 mL.

2.4 | Nanofiber AIEX Protocol

All chromatography experiments were conducted on an ÄKTA Avant 150 (Cytiva). Frozen CCH LV-GFP material (p24 concentration = 0.44 $\mu\text{g/mL}$) was thawed rapidly in a water bath at 37°C prior to chromatography. Studies indicating similarity between fresh and freeze-thawed CCH starting material during AIEX can be found in the PhD thesis of G. Pamenter (Pamenter 2025). 4 process buffers were used for this work, with all formulated in 20 mM Tris, pH 7.2 at NaCl concentrations: 0 mM (Dilution, Buffer D), 50 mM NaCl (Buffer A), 2000 mM NaCl (Buffer B) and 100 mM for diafiltration buffer (DF Buffer). Prior to processing ÄKTA systems and nanofiber devices were subject to a decontamination in 0.5 M NaOH for 1 h followed by system equilibration in Buffer A. Finally, Q-Nanofibers were charged with Buffer B then equilibrated in Buffer A. For DoE runs, 50 CV of thawed CCH was diluted 1:1 in Buffer D and loaded at 50 CV/min (11 mL/min). Following a 50 CV Buffer A flush, linear gradient elution was performed by mixing Buffer A and B up to 66.7% Buffer B content (50–1350 mM NaCl).

Fractions were collected every 5 CV (1.1 mL). Extended contact times were achieved using a static “on-column” incubation step following the wash, as described in Pamerter et al. (2023). Static incubation times of 0 min (giving an average contact time = 3 min), 23.5 min (contact time = 26.5 min), and 47 min (contact time = 50 min) were implemented.

2.5 | HEK293T and T-Cell Transduction Assays

$$\text{Titre} \left(\frac{\text{TU}}{\text{ml}} \right) = \frac{[\% \text{ cells expressing GFP} \times \text{number of cells at transduction} \times \text{dilution factor}]}{\text{Volume of Vector added at transduction}} \quad (1)$$

Functional titer was determined using a HEK293T transduction assay. Samples were diluted in DMEM Media (Sigma-Aldrich, Merck) supplemented with polybrene (Sigma-Aldrich, Merck) and added in duplicate to adherent HEK293T cells. Cell analysis was performed using an Attune NxT acoustic focusing flow cytometer (Thermo Fisher Scientific), collecting size and GFP fluorescence data to determine the percentage of cells exceeding the background fluorescence of non-transduced cells. GFP titer was calculated by Equation (1):

For measurement of T cell functional vector transduction, Peripheral Blood Mononuclear Cells (PBMCs) within the OXB PBMC bank, isolated from Leukapheresis from two donors, subsequently referred to here as Donor 1 and Donor 2, were employed in this study. Following T-cell activation, vector samples were diluted in media and then added to the activated cells. The total vector volume was 100 μL . “Transduction volume” corresponds to the amount of this 100 μL comprised of the vector. For example, a transduction volume = 6.25 μL means 93.75 μL of media and 6.25 μL of vector. 10 μM Nevirapine was also added to additional replicates as a control for pseudo-transduction. Analysis was performed on a Fortessa X-20 flow cytometer (BD Biosciences). Lymphocyte populations were gated according to their forward and side scatter properties. Following subsequent singlet and dead cell identification, live CD3^+ T cells expressing GFP were identified ($\% \text{CD3}^+ \text{GFP}^+$).

2.6 | p24 Concentration Measurement and Total Particle Recovery Calculation

HIV-1 p24 concentration was measured via the Ella (Protein Simple) high-throughput automated HIV-1 p24 enzyme-linked immunosorbent assay (ELISA), developed in-house by OXB. To determine the overall cumulative total particle recovery for each nanofiber run, p24 concentration of each individual elution fraction was summed to calculate “total eluted p24 mass” and used in Equation (2). As not all p24 is vector-associated and significant quantities of “free” p24 are present in the AIEEX flowthrough, only the recovery of bound p24 mass is considered here.

$$\begin{aligned} \text{Total Particle Recovery} \\ = \frac{\text{Total Eluted p24Mass } (\mu\text{g})}{\text{Total Loaded p24mass } (\mu\text{g}) - \text{Flowthrough p24mass } (\mu\text{g})} \quad (2) \end{aligned}$$

2.7 | Design of Experiment and MLR

A design of experiment (DoE) methodology was employed to systematically assess the impact of nanofiber design factors and contact time. A central composite design (CCD) with two center points was applied to measure the effect of three factors at three levels: contact time (3, 26.5, and 50 min), fiber diameter (400, 500, and 600 nm), and membrane porosity (0.4, 0.5, and 0.6 v/v). This yielded 16 experimental runs, inclusive of replicate center points. Manufacturing constraints prevented the inclusion of ligand density as a CCD factor at 3 levels. Thus, the whole 16 run DoE was duplicated at two ligand densities (564 and 272 $\mu\text{mol/g}$), giving 32 experimental runs in total. A comprehensive description of this workflow is provided in Figure 1B. A complete list of runs and their associated experimental factor setting is given in the Sup. Figure 2.

$$\begin{aligned} y = & \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{1,2} X_1 X_2 + \beta_{1,3} X_1 X_3 \\ & + \beta_{1,4} X_1 X_4 + \beta_{2,3} X_2 X_3 + \beta_{2,4} X_2 X_4 + \beta_{3,4} X_3 X_4 + \beta_{1,1} X_1^2 \\ & + \beta_{2,2} X_2^2 + \beta_{3,3} X_3^2 + \varepsilon \quad (3) \end{aligned}$$

y = Response variable, β = regression coefficient, X_1 = Porosity, X_2 = Nanofiber Diameter, X_3 = Contact Time, X_4 = Ligand Density ε = random error.

For MLR a second order polynomial equation, including one factor interactions, was employed as the base model structure. The general form is given in Equation (3), with the second-order term for ligand density excluded. MLR was conducted using JMP 18 Statistical software (SAS), “fit model” platform. Selection of statistically significant terms was conducted via “stepwise” regression using forward and backward selection with a corrected Akaike's Information Criterion (AICc) criteria and model hierarchy enforced. Final models were constructed using “standard least squares” regression, with effect magnitudes determined from the regression coefficients of coded factors.

2.8 | Fitting of Bimodal Skewed-Gaussian Model and Elution Profile Simulations

To extract shape features for each LV subpopulation's elution, the two distinct peaks in the “two-peak” profile were each modeled using a Gaussian function with defined skew to capture the non-normal behavior in their probability density functions. The overall elution profile is then obtained by summing these two skewed-Gaussian distributions, with each distribution corresponding to a single LV subpopulation Equations (4,5). This bimodal skewed-Gaussian model was fit to data via nonlinear least squares fitting with SciPy's “optimize. curve_fit” function in Python.

$$q_1 = \frac{B_1 \varphi(X_1) \cdot 2\Phi(\alpha_1 X_1)}{\Phi(0)} + \frac{B_2 \varphi(X_2) \cdot 2\Phi(\alpha_2 X_2)}{\Phi(0)} \quad (4)$$

$$\begin{aligned} \varphi(X) &= \frac{1}{s_1 \sqrt{2\pi}} e^{-\frac{X^2}{2}}, \Phi(X) = \frac{1}{2} \left[1 + \text{erf} \left(\frac{X}{\sqrt{2}} \right) \right], \\ X &= \frac{x - m}{s} \quad (5) \end{aligned}$$

where $\varphi(X)$ is the normal distribution probability density function and $\Phi(X)$ normal cumulative distribution function defined by:

Where q_1 = eluted viral concentration (p24 $\mu\text{g/mL}$), $B_{1,2}$ = Peak Magnitude Coefficient (μg), $s_{1,2}$ = peak $1/2$ standard deviation (mL), $m_{1,2}$ = Peak $1/2$ mean (mL), x = volume (mL), $\alpha_{1,2}$ = skewness factor and $\Phi(0)$ = cumulative distribution function at $\alpha_{1,2} = 0$.

To visualize how nanofiber structural factors (DoE factors) influence the shape of the LV elution profile, a separate MLR model was fit to each of the eight skewed-Gaussian shape parameters (i.e., eight responses). These fitted models were then used to reconstruct predicted elution profiles as a function of DoE factors, including factor combinations not directly tested experimentally. This allowed us to visualize how incremental changes in nanofiber structure influence peak-specific elution and overall profile shape in a continuous manner (Figure 8). To generate confidence intervals for our simulated elution curves at each factor setting, we quantified how uncertainty in the fitted linear-regression coefficients propagated into the simulated elution profiles. This was achieved via residual bootstrapping, a technique routinely employed for both univariate and multivariate linear regression (Freedman 1981; Eck 2018; Ayoade and Loinmi 2019). In brief, the original linear regression fits for the eight skewed-Gaussian parameters produced a 32×8 residual matrix (runs $\times$ responses). A new pseudo-response was then generated for each DoE factor setting by taking the mean prediction from the original fitted polynomial equations and adding a resampled residual. This was achieved by resampling row vectors from the residual matrix with replacement, so that for each DoE condition, all eight skewed-Gaussian parameters sampled their residuals from the same run in each bootstrap iteration, preserving the residuals correlation structure between skewed-Gaussian parameters. MLR was then performed on these pseudo-responses to obtain new bootstrap estimates of the regression coefficients, which were used to calculate a new skewed-Gaussian parameter set and subsequently reconstruct a new elution curve for the given DoE factor setting. This process was repeated 2000 times per DoE factor setting, generating a distribution of 2000 candidate elution curves from which 95% confidence bands were defined.

3 | Results and Discussion

3.1 | T-Cell Transduction Efficiency of Peak 1 and Peak 2 LV

In our previous work, preliminary T cell studies showed that LV eluted in the strongly interacting peak 2 (glycosaminoglycan binding, Figure 2Ai) had ~ 2 -fold lower transduction efficiency than the weaker binding peak 1 (envelope/protein binding), paralleling findings of Yamada et al. (2003) in murine hematopoietic stem/progenitor cells (Pamenter et al. 2024; Yamada et al. 2003). However, those studies assessed only neat AIEF fractions (i.e., not further purified) at a single MOI = 0.5, leaving questions of impurity effects and dose dependence unresolved. In CAR-T manufacturing, differing doses are chosen for specific products to balance higher transduction efficiency against the negative effects of higher dosing, such as

increased cost, T-cell exhaustion, and risk of multiple integration events (Dan and Kang-Zheng 2025; Ho et al. 2021). First, we wished to strengthen the analysis of subpopulation performance differences to better define what the aims of the nanofiber design should be (i.e., isolate peaks, or collect both). To do this, Peak 1 and 2 eluates were further purified, using UF/DF, then used to transduce T-cells at increasing volume. This was done in biological triplicate. Transduction volume was used over MOI as comparable HEK293T titers were achieved for Peak 1 and Peak 2 ($\sim 2.35 \times 10^7$ TU/mL), and because direct use of volume prevents uncertainty in the HEK293T titer biasing the T cell transduction dosing. For reference, a $10 \mu\text{L}$ transduction volume corresponds to an MOI of ~ 2.75 .

Figure 2B,C show the %CD3⁺GFP⁺ T-Cells and their viability at harvest for two patient donor cells. At low transduction volume ($\leq 6.25 \mu\text{L}$) Figure 2B shows significantly higher transduction efficiency in Peak 1 (48% donor averaged CD3⁺GFP⁺ T-Cells, $3.1 \mu\text{L}$) than Peak 2 (19%). This gives a 2.53-fold increase (p -value = 1.91×10^{-7}) in T-cell transduction efficiency for Peak 1 LV at this dose ($\sim$ MOI = 0.85), which is consistent with our prior findings. However, Peak 1 achieves an earlier maximum % CD3⁺GFP⁺ T-Cells of 56% (Donor Averaged at $6.25 \mu\text{L}$, $\sim$ MOI = 1.7), then begins to decline with increasing transduction volume. In contrast, although Peak 2 appears significantly less potent at lower transduction volume, a sustained rise is seen across the experimental range with no maximum obtained even at $50 \mu\text{L}$ ($\sim$ MOI = 13). As such, significantly higher donor averaged maximum %CD3⁺GFP⁺ T-Cells are obtained for Peak 2 (69%) when compared with Peak 1 (56%, p -value = 5.77×10^{-3}). Importantly, Figure 2C shows a secondary effect of Peak 1, where increasing volume results in a sustained reduction in T-cell viability from 90.5% (Donor Averaged, $0.3 \mu\text{L}$) to 10.2% ($50 \mu\text{L}$). This is a large decline in T-cell viability upon treatment with Peak 1 LV, which is not observed in Peak 2, and has obvious implications for LV product efficacy.

3.2 | Impact of Vesicle Impurities on Peak 2 LV Transduction Efficiency and Viability

In our prior work, we demonstrated that Peak 1 has a significantly higher ratio of VSV-G to p24 compared to Peak 2 (Figure 2Aii). One potential reason for this reduction in peak transduction efficiency and viability at higher doses is the presence of particulate species that compete with LV for T-Cell entry and promote T-cell exhaustion. VSV-G vesicles (extracellular vesicles containing the LV envelope pseudotype) have been shown to form during VSV-G transfection in HEK293T cells and are sufficiently large (~ 100 nm) to be retained during 500 kDa TFF (Mangeot et al. 2011). Elevated VSV-G levels in Peak 1 LV could stem from the presence of these species. Under conditions of competitive receptor binding (i.e., high doses) the high VSV-G expression in these particles could lead to them out-competing LV for LDL-Receptor binding sites, driving preferential binding and internalization by T cells.

To assess the potential impact of VSV-G vesicles on T-cell transduction, we generated HEK293T CCH in the absence of LV structural plasmids (Figure 3A). These CCHs were subsequently

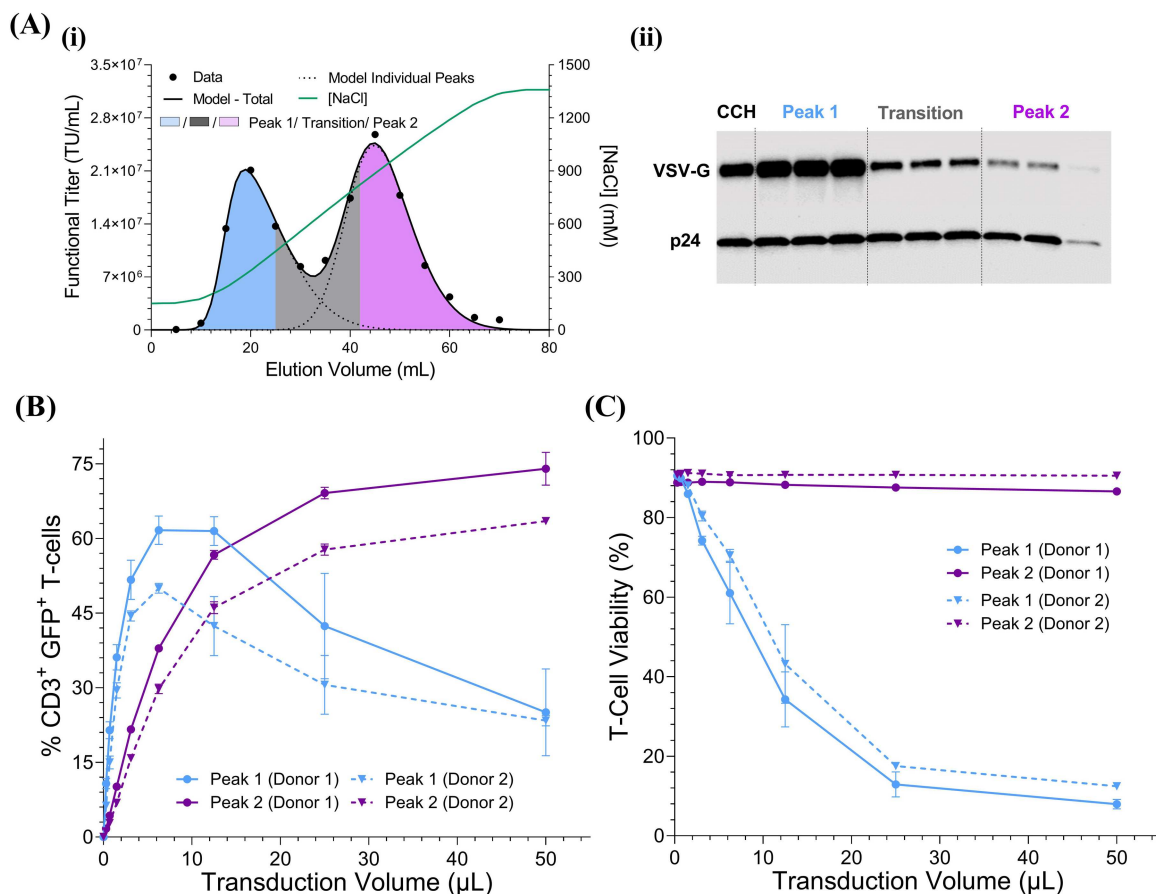


FIGURE 2 | Transduction of primary T cells using purified LV from Peak 1 and Peak 2. Error bars represent ± 1 SD for $N = 3$ biological replicates unless otherwise stated. Peak materials were generated from the two-peak AIEX gradient elution profile using the same CCH material described in Pamerter et al. (2024). The total volume of added sample was 100 μ L. “Transduction volume” corresponds to the amount of this 100 μ L comprised of vector. For example, a transduction volume = 6.25 μ L means 93.75 μ L of media and 6.25 μ L of vector. In relation to MOI, a 10 μ L transduction volume corresponds to an MOI of ~ 2.75 . (A) The two-peak AIEX elution profile indicating the regions used to generate Peak 1, Transition, and Peak 2 LV fractions (i) and the normalized VSV-G concentration in each fraction as determined by western blot (ii). Data is reproduced directly from Pamerter et al. (2024) for clarity. (B) Percentage of CD3⁺GFP⁺ T cells transduced with purified Peak 1 or Peak 2 LV at increasing transduction volumes in two donors. (C) Viability of transduced T cells at harvest following transduction with purified Peak 1 or Peak 2 LV at increasing transduction volumes in two donors.

purified to generate concentrated particle stocks, which we refer to as vesicle preparations, for spiking into T-cell transduction assays. It is acknowledged that this procedure will generate a mixture of particle types, and the term “vesicle” is used here as a holistic label that avoids confusion with LV particles. For VSV-G-positive vesicle preparations ([+VSV-G Vesicles]), HEK293T cells were transfected with pVSV-G and pBluescript. pBluescript was used as a non-coding stuffer plasmid to match pDNA levels used in LV production. In parallel, VSV-G-negative vesicles ([−VSV-G Vesicles]) were produced by transfecting HEK293T cells with pBluescript alone. This yielded two vesicle CCH materials, (+)VSV-G and (−)VSV-G.

Because Peak 1 and Peak 2 regions of the LV profile differed in cytotoxicity, we explored the T-Cell inhibitory effects of vesicle materials eluted from each of these AIEX peak regions. Each of the (+) and (−) VSV-G CCH materials was therefore fractionated on Q-membranes into Peak 1 and Peak 2 before concentration/diafiltration using a 500 kDa TFF. A linear AIEX gradient was applied to (+)VSV-G CCH to identify [NaCl] conditions that separate Peak 1 and Peak 2 vesicles (Figure 3C).

This yielded UV280 elution profiles with notable similarity to LV (Figure 3B) and a two-peak profile for VSV-G concentration (Figure 3C) was also observed. This could suggest two sub-populations of VSV-G particle that also differ in their glycosaminoglycan (GAG) binding, as we previously showed for LV (Pamerter et al. 2024). A 700 mM NaCl cut was selected to elute Peak 1 and Peak 2 vesicles. Following UF/DF, 4 vesicle preparations were generated: (+)VSV-G Vesicles - Peak 1, (+)VSV-G Vesicles - Peak 2, (−)VSV-G Vesicles - Peak 1, and (−)VSV-G Vesicles - Peak 2.

For T-cell transduction studies, a fixed volume of Peak 2 LV (5 μ L) from the linear range of the titration curve in Figure 2B was added alongside increasing volumes of purified vesicles (0–50 μ L). The total volume added during transduction, including vector, vesicles, and media, was maintained at the usual 100 μ L. Figure 3D,E give the %CD3⁺GFP⁺ and T-cell viability profiles for Donor 1 (i) and Donor 2 (ii). Increasing Peak 1 and Peak 2 (+)VSV-G vesicle volume caused a sustained drop in the %CD3⁺GFP⁺, reducing the %CD3⁺GFP⁺ from 24.6% (donor averaged, 0 μ L) to 3.83% (50 μ L) (Figure 3Di,ii). These

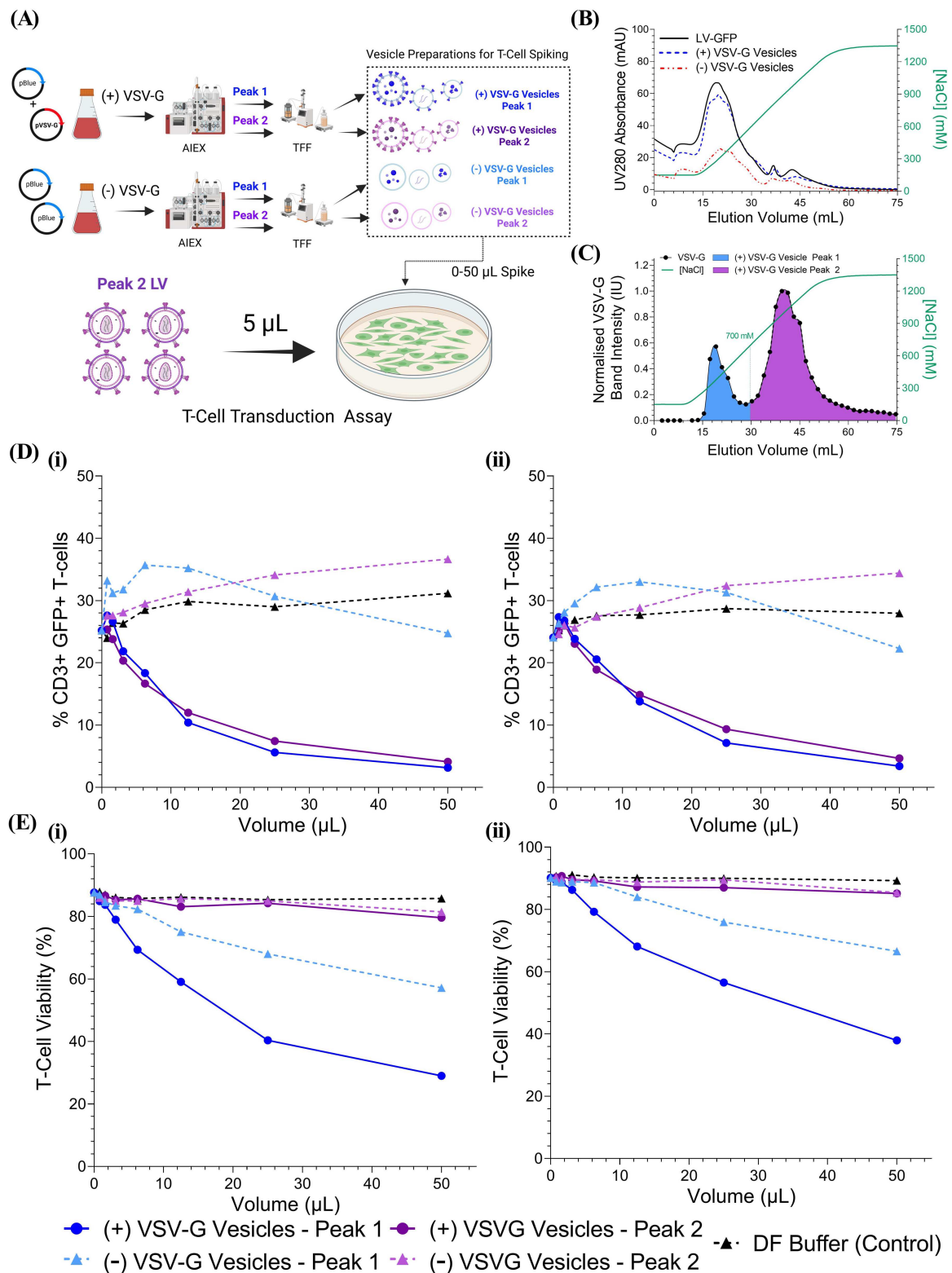


FIGURE 3 | Impact of (+/-) VSV-G vesicle spiking on T-Cell transduction efficiency of Peak 2 LV. In this figure, “vesicle” refers to all particles purified from HEK293T cell culture. A mixture of particle types is present, but the term “vesicles” is used as a general label that avoids conflation with LV particles. (A) Schematic representation of vesicle production and T-cell spiking. T-cells were transduced with a fixed quantity (5 μ L) of Peak 2 LV and spiked with increasing volumes of purified vesicle material (0–50 μ L). A DF buffer control was also included. Vesicles were generated by culturing HEK293T cells in the presence of a pBluescript plasmid only [(-) VSV-G] or pBluescript and pVSV-G together [(+) VSV-G]. This was followed by AIEX fractionation to produce Peak 1 and Peak 2 vesicle materials. Peaks were subsequently ultrafiltered/diafiltered. Single biological replicates are shown. (B) Chromatogram showing similarity in gradient elution profiles of LV-GFP CCH and (+/-) VSV-G Vesicle CCH. (C) Gradient elution profile of VSV-G concentration assessed semi-quantitatively by Western blot band intensity. The 700 mM cut-point used to elute Peak 1 (< 700 mM) and Peak 2 (> 700 mM) is indicated. (D) Percentage of CD3⁺GFP⁺ T cells transduced for donor 1 (i) and donor 2 (ii). (E) Viability of transduced T cells for donor 1 (i) and donor 2 (ii).

data imply an inhibitory effect of (+)VSV-G Vesicles on T-cell transduction efficiency in both Peak 1 and Peak 2. Although both (+)VSV-G vesicles from Peak 1 and Peak 2 caused a reduction in %CD3⁺GFP⁺, only the (+)VSV-G Vesicles eluted from Peak 1 caused a reduction in T cell viability from 88.9% to 33.5% (donor averaged) from 0 to 50 μ L (Figure 3Ei,ii). Conversely, no notable reduction in viability was observed for (+) VSV-G in Peak 2. Unlike (+)VSV-G vesicles, Peak 1 and Peak 2 (-)VSV-G vesicles caused no reduction in %CD3⁺GFP⁺ T cells when increasing their volume (Figure 3Di,ii). In fact, a slight increase in GFP-positive cells may be observed at higher doses for (-)VSV-G vesicles in Peak 2, but more data is needed to confirm this observation. Despite no clear effect on % CD3⁺GFP⁺, (-)VSV-G vesicles from Peak 1 also had a strong impact on T-cell viability, decreasing it from 88.9% (0 μ L) to 61.9% (50 μ L) (Figure 3Ei,ii). As observed for (+) VSV-G vesicles, (-)VSV-G vesicles from Peak 2 had no significant impact on T-cell viability.

These data show that all purified HEK293T derived particles containing VSV-G, referred to as (+)VSV-G vesicles here, significantly inhibit the transduction efficiency of LV in a dose-dependent manner. As (-)VSV-G vesicles in Peak 1 did not impede the number of GFP-positive cells, but significantly reduced cell viability, these data also suggest that Peak 1 contains a cytotoxic component not found in Peak 2. The presence of VSV-G also seems to exacerbate the effect of this, or these, cytotoxic species. Combined, these results indicate that the lower transduction efficiency and T-cell viability observed for Peak 1 LV at higher doses (Figure 2) is likely caused by co-elution of VSV-G-containing vesicles. This creates a challenge for LV chromatography adsorbent design, as maximizing particle recovery requires collecting the full elution pool, favoring adsorbents that produce tighter, more homogenous LV elution profiles. However, this same strategy likely increases co-elution of detrimental impurities that impair transduction efficiency and T-cell viability.

We propose that future LV AIEX processes may choose to exclude Peak 1 LV and instead isolate, or increase the quantities of, the more potent Peak 2 population, provided high enough recovery can be achieved. Our nanofiber design aims reflected this. We therefore set an adsorbent design goal of maximizing LV recovery whilst simultaneously increasing Peak 1-Peak 2 separation so that Peak 2 LV can be collected in isolation. Nonetheless, in low-dose cases, Peak 1 LV showed significantly higher %CD3⁺GFP⁺ T Cells, which is likely favorable for some LV products. As such, this work has the broader aim of understanding how Q-nanofiber design influences Peak 1-Peak 2 separation, and thus selectivity.

3.3 | Designing Nanofiber Adsorbent Structures for Improved Lentiviral Vector Recovery and Peak Separation

To model how nanofiber structure influenced LV elution profiles, a “functional” DoE was conducted. Nanofiber Porosity (0.4, 0.5, and 0.6 v/v), nanofiber diameter (400, 500, and 600 nm), and contact time (3, 26.5, and 50 min) were varied in a 16 run DoE with each run generating a functional (not scalar)

response (i.e., the linear gradient elution profile for p24 concentration). This “functional” DoE was repeated at two ligand densities (272 and 564 μ mol/g) giving 32 runs total. For conditions that weakened binding strength, if not properly underloaded there is the possibility that this could lead to changes to the elution profile from differences in competitive binding and not adsorbent structure. To generate elution data sets that isolated the impact of adsorbent changes from differences in competitive binding effects, Q-nanofibers were significantly underloaded with 50 CV of diluted LV-GFP CCH. These conditions were defined from initial scouting studies (Sup. Figure 1) which showed no LV breakthrough up to a loading of 150 CV. Figure 4A gives a representative DoE chromatogram. The full experimental workflow is given in Figure 1B.

3.4 | Impact of Nanofiber Structure on Overall Cumulative Recovery of LV

Due to low process yields, high overall vector recovery remains a key goal for LV AIEX capture. To evaluate the influence of nanofiber factors on overall AIEX recovery, cumulative total particle recovery was calculated by summing the p24 mass in all gradient elution fractions for each DoE run. Although we acknowledge that functional particle recovery would be a preferable metric to total particle recovery, the volume of samples and inherent variability of cell-based assays across different fraction NaCl concentrations made accurate analysis of functional vector recovery impractical. Instead, we chose to base our nanofiber design on p24 concentration data and then validate functional recovery in the selected Nanofiber later (Section 3.8).

Figure 4B gives the raw data for cumulative total particle recovery alongside an MLR model fit to the data (Figure 4C,D). As expected, time spent in the adsorbed state (contact time) was the primary factor affecting cumulative particle recovery, aligning with our observations for LV in Q-membrane adsorbents and those of Ad5 purification in nanofiber adsorbents (Pamenter et al. 2023; Turnbull et al. 2019). Porosity emerged as the only other factor influencing recovery with higher porosity giving higher recovery but with diminishing returns. The magnitude of LV loss with contact time was also exacerbated at low porosity, reducing from 59% ($t = 3$ min) to 24% ($t = 50$ min) at porosity = 0.4 and from 73% ($t = 3$ min) to 37% ($t = 50$ min) at porosity = 0.6. This gives a modeled reduction of 2.5- and 1.95-fold across the time range (porosity = 0.4 and 0.6, respectively).

These results support the initial hypothesis that lower-porosity adsorbents increase time-dependent LV loss, perhaps due to simultaneous binding of multiple fibers (Figure 1Aiii). However, this is likely an oversimplification. In reality, Nanofiber manufacturing settings result in unique 3D structures that may influence recovery in more complex ways. The high compression needed for lower porosities creates smaller, more heterogeneous pores whilst causing notable fiber fusion (Ovari et al. 2024). This structural heterogeneity could lead to formation of imperfections such as highly charged “pockets” where numerous fibers collect to form high-avidity zones. LV entering these pockets may undergo immediate avidity and irreversible binding, thus lowering recovery. Alternatively, this porosity

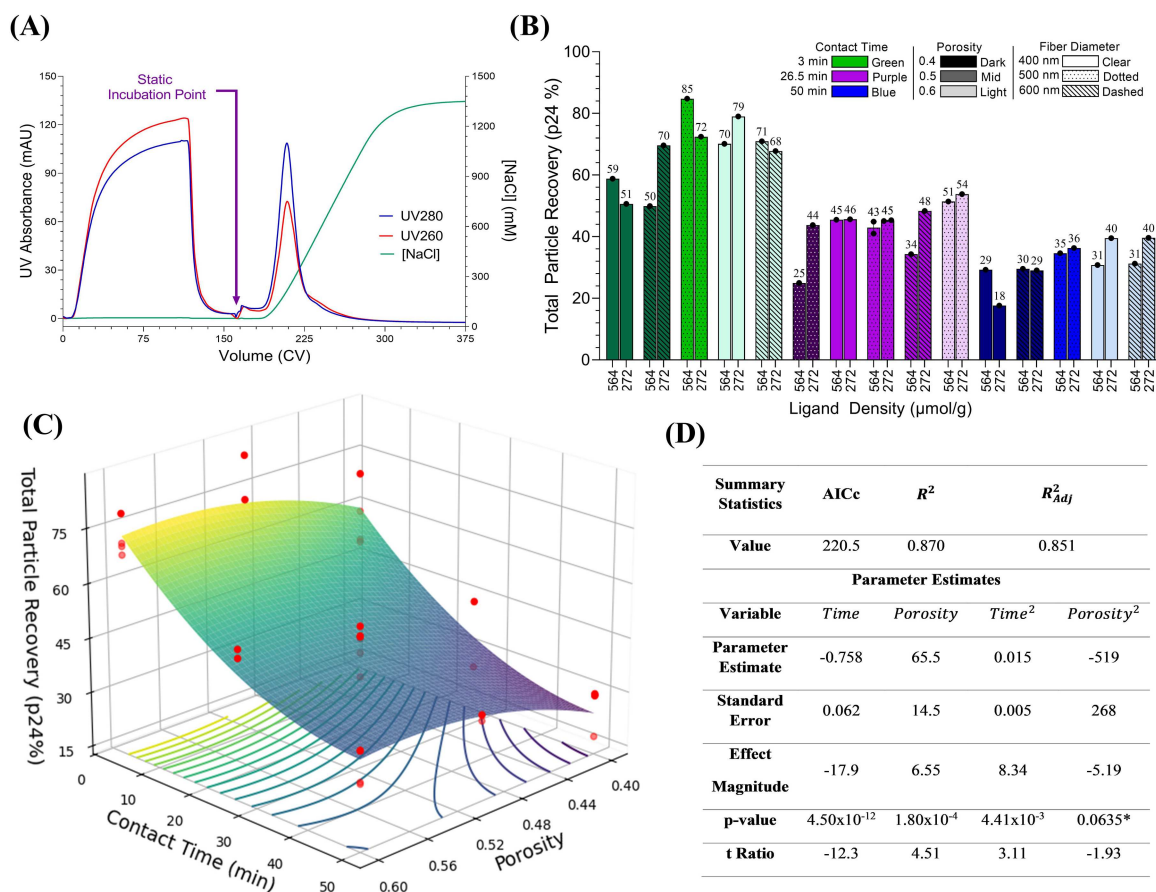


FIGURE 4 | Impact of Q-nanofiber structure and contact time on cumulative total particle recovery (recovery of bound p24). (A) Representative chromatogram from a single DoE run, with the on-column incubation point indicated. The condition shown is (porosity = 0.6, diameter = 600 nm, contact time = 3 min, ligand density = 564 μmol/g). Chromatogram volume is given as the absolute loaded volume. Thus, a loaded CCH volume of 100 CV (post-dilution) corresponds to a 50 CV pre-diluted CCH load. (B) Raw data plot of cumulative total particle recovery for all DoE conditions. (C) Multilinear regression (MLR) model for cumulative total particle recovery, with the final model overlaid on the raw data in red and a contour plot projected onto the lower axis surface. (D) Summary statistics for the regression model. Stepwise regression was performed using AICc selection criteria. Effect magnitude is represented by the coded factor parameter coefficients. * Model parameters selected by AICc but with $p > 0.05$.

effect could result from physical entrapment of LV particles, as is observed in monolithic chromatography adsorbents (Schimek et al. 2025). However, the mean pore size of the fibers used in this study was ~300–400 nm at low porosity, with only a small increase observed for fibers with high porosity (~400–450 nm). These pores should, in theory, allow LV to pass through. However, an explicit non-binding DoE would be required to completely rule out this hypothesis.

Interestingly, ligand density was not identified as a significant factor impacting recovery. This observation does not fit the hypothesis in Figure 1Aii and contrasts with similar experiments on the time-dependent recovery of Ad5 in Q-Nanofiber AIEC (Turnbull et al. 2019). However, Turnbull et al. explored higher ligand density values (440–1029 μmol/g), noting little impact of contact time at 440 μmol/g. The lower range used here (272–564 μmol/g) may be too small to see a notable shift in recovery. Nevertheless, the broad range of recoveries attained in this work (18%–85%) underscores the critical importance of nanofiber design and the benefits of fast processing in achieving high LV-AIEC yields.

3.5 | Lentiviral Vector Gradient Elution Profile Raw Data

Figure 5 gives the p24 elution profiles from each DoE run. A wide variety of profile shapes are obtained. Some runs exhibit the characteristic two-peak elution behavior (e.g., Figure 5C, 564 μmol/g), with others resembling single shouldered peaks (e.g., Figure 5G, 272 μmol/g), or even single peaks (e.g., Figure 5N, 272 μmol/g). Notable peak tailing is also seen in most runs (>900 mM NaCl). This raw data inspection highlights the large variety of LV elution profiles obtained when varying the adsorbent structure. These data may explain why previous authors, despite observing broad salt elution ranges, do not always report two clear peaks (Ghosh et al. 2022). As identical feed material is used in Figure 5, and because we previously demonstrated the two peaks stem from subpopulations of LV already present in the CCH (Pamenter et al. 2024), absence of two distinct peaks must therefore result in varying degrees of peak overlap brought about by adsorbent structural changes. We therefore sought to model the adsorbent features that brought about these changes in population overlap, herein referred to as “Peak Separation.”

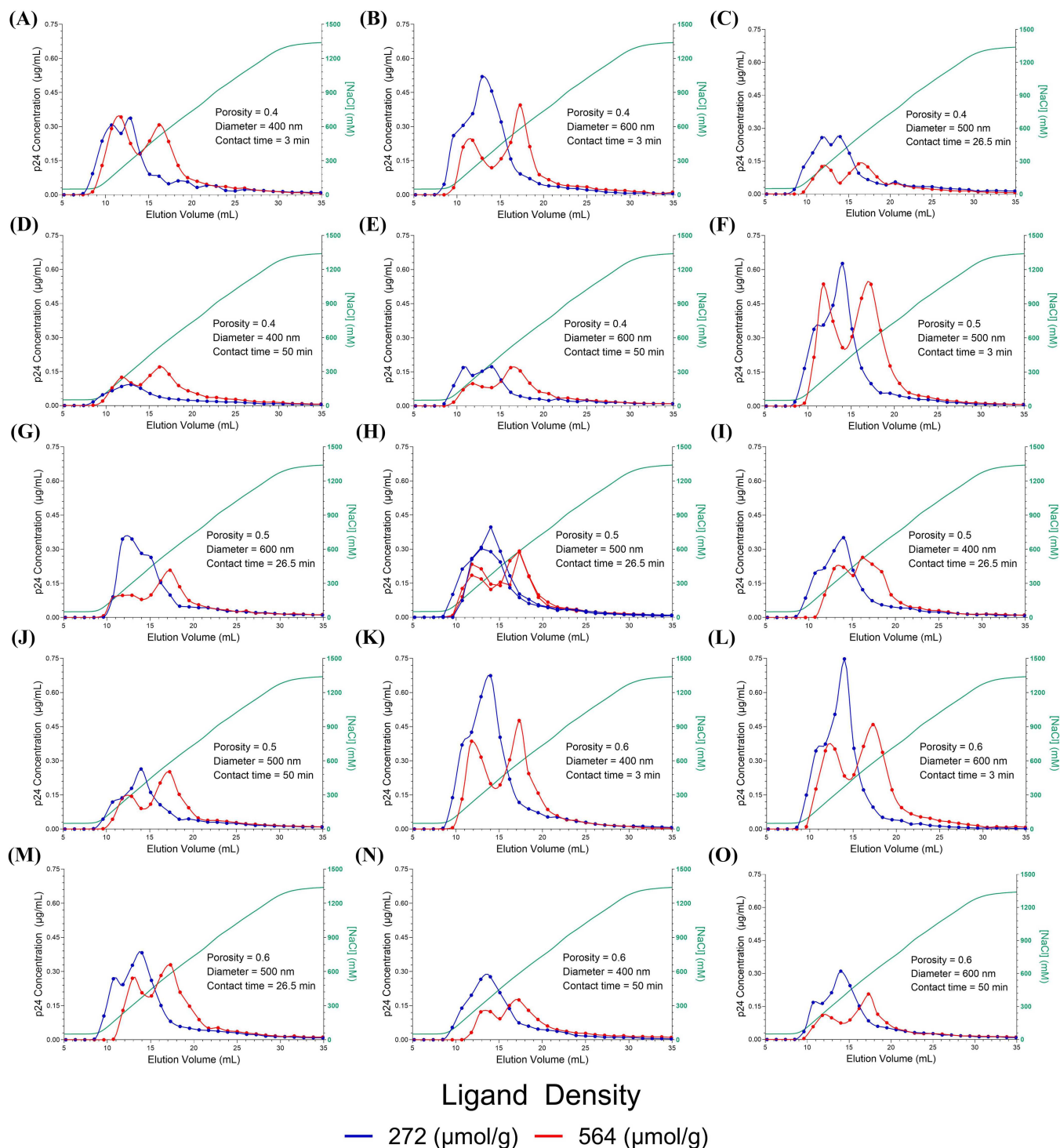


FIGURE 5 | Complete gradient-elution raw data set. Smoothing splines were applied to generate continuous elution profiles. Panels represent individual DoE runs, with the center-point condition displayed in the middle panel. Both center-point replicates are shown on the same panel with individual runs plotted to indicate run to run variability. Data for both ligand densities (564 and 272 $\mu\text{mol/g}$) are presented within each panel. Corresponding DoE factor settings are given on each figure (A–O).

3.6 | Modeling the Impact of Nanofiber Design on LV Elution Profile and Peak Separation

Due to the multivariate nature of the data, the individual impact of DoE factors on LV Peak Separation is not immediately apparent. To address this, we applied a functional DoE modeling approach (A PCA was also conducted to assess major causes of profile variability by a different approach, and can be found in

supp figure 3). To model the changes in elution profile shape, a bimodal skewed-Gaussian model Equation (4) was first fit to each LV elution profile to estimate peak specific elution properties such as Peak 1 and Peak 2 retention (e.g., $\mu_{1\&2}$) (Figure 6). The values obtained for these skewed-Gaussian parameters were then used as new response variables for MLR, with a polynomial equation generated for each of them. This allowed us to quantify

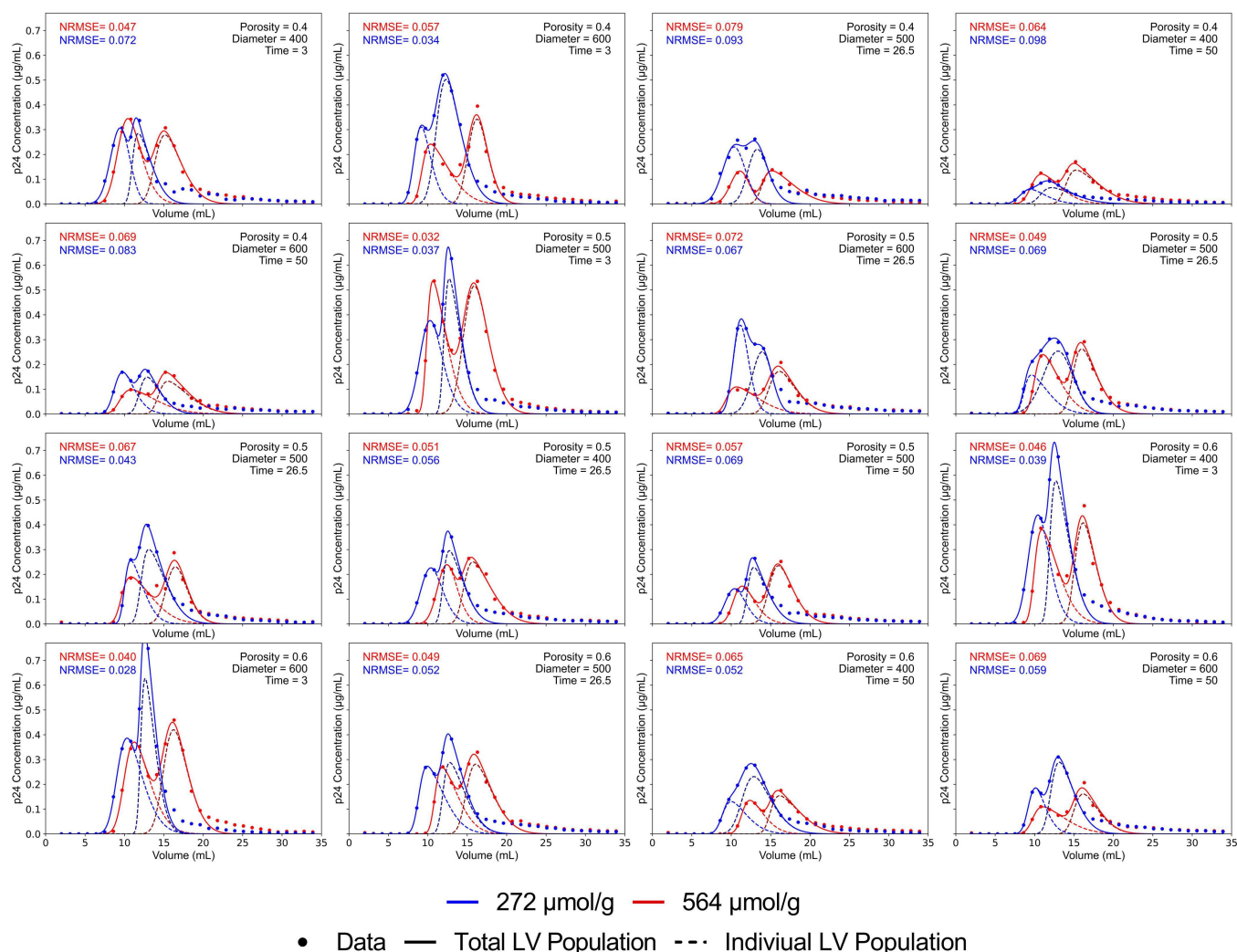


FIGURE 6 | Fitting of bimodal skewed-Gaussian model to the p24 elution profile raw data for each experimental run. Data for both ligand densities, 564 µmol/g (red) and 272 µmol/g (blue), are shown on each subplot. Normalized Root Mean Square Error (NRMSE) values are reported on the figure to indicate general goodness of fit. Data from the “Tail” region were excluded during fitting to better characterize the main elution peak region. Center-point replicates are displayed individually, as model fits were applied separately to each replicate to generate two independent parameter sets at the replicate center points.

the impact of nanofiber design on LV elution profile shape. As a main nanofiber design goal was to identify an adsorbent capable of isolating the Peak 2 LV subpopulation, we define “Peak Separation” as the key response for this (i.e., $\mu_2 - \mu_1$, Δ mM NaCl). This Peak Separation model is given in Figure 7A, alongside actual vs model predicted values in Figure 7B, demonstrating a reasonable degree of fit ($R^2 = 0.74$, $R^2_{adj} = 0.72$).

Ligand density emerged as the primary factor influencing peak separation (magnitude = 59.3), increasing from 138 ± 27 ΔmM (ligand density = 272 µmol/g, diameter = 400 nm) to 256 ± 27 ΔmM (ligand density = 564 µmol/g, diameter = 400 nm). A significant impact of nanofiber diameter was also observed (p -value = 7.69×10^{-3}). The magnitude of this effect (magnitude = 25.1) was approximately half that of ligand density and resulted in an increase in peak separation from 138 to 188 ΔmM NaCl when increasing the diameter from 400 to 600 nm (at ligand density = 272 µmol/g). The magnitude of this diameter effect is perhaps surprising, as increasing it by just 50% produced approximately half the response observed from doubling the ligand density.

This supports the broad claims presented in Figure 1Ai, suggesting that decreased surface curvature (increased fiber diameter) increases binding strength. By manipulating both nanofiber diameter (400–600 nm) and ligand density (272–564 µmol/g), this model implies that LV peak separation can be increased from 138 ± 27 ΔmM (95% CI) to 306 ± 27 ΔmM NaCl. However, a significant amount of unexplained variation is observed in the model. Critically, when combined with Figure 4 these two models suggest that peak separation and recovery are controlled by separate adsorbent features. This is beneficial for nanofiber design as it implies that Peak Separation can be tuned without compromising LV recovery.

3.7 | Defining Sources of Increased Peak Separation and Selection of Q-Nanofiber Structure by Simulating LV Elution Profiles

Nanofiber diameter and ligand density were shown to increase Peak Separation. However, the causes of this change are not immediately apparent from Figure 7 alone. Because the two LV

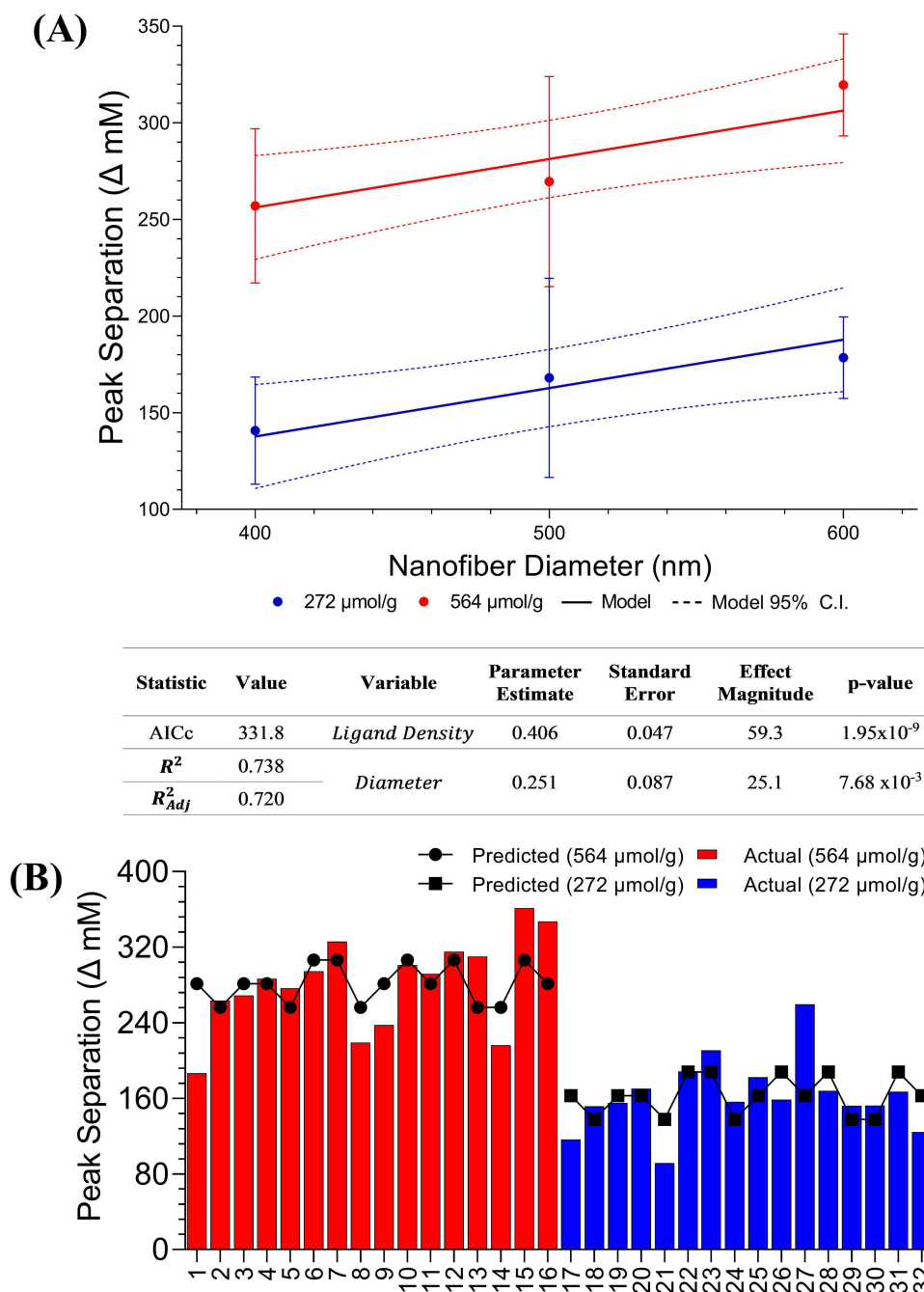


FIGURE 7 | Multilinear regression model for the impact of ligand density and nanofiber diameter on LV subpopulation peak separation ($\mu_2 - \mu_1$) during gradient elution on Q-nanofibers. (A) One-dimensional model plot and summary statistics for peak separation ($\mu_2 - \mu_1$). Nanofiber diameter is shown on the x-axis, with ligand density represented as two separate lines. Error bars indicate ± 1 SD of the experimental peak-separation data; these reflect raw data variability around the model and not model confidence, which is shown by the dashed 95% confidence-interval lines. (B) Actual vs predicted peak-separation values for each individual run. X-axis gives run number, as detailed in Sup. Figure 2.

subpopulations are thought to bind through distinct envelope species, they may respond differently to changes in adsorbent structure. For example, the observed increase in peak separation could arise from greater retention of Peak 2, reduced retention of Peak 1, or a combination of both. LV elution profiles were therefore simulated to visualize this (Figure 8).

Simulations were run for increasing ligand densities (left to right) and nanofiber diameters (top to bottom) at a fixed porosity (0.6) and contact time (3 min). At low ligand density

and diameter (top-left panel), this model predicts substantial overlap between LV subpopulations (peak separation = 138 Δ mM). This limited resolution between peaks makes the nanofiber structure unsuitable for isolating Peak 2. Furthermore, this model suggests the primary driver of increased peak separation with increasing ligand density is a shift of Peak 2 LV toward higher elution [NaCl], with increasing nanofiber diameter producing a similar effect. Thus, the enhanced separation associated with higher ligand density and larger diameter arises almost entirely from increased retention of Peak 2, with Peak 1

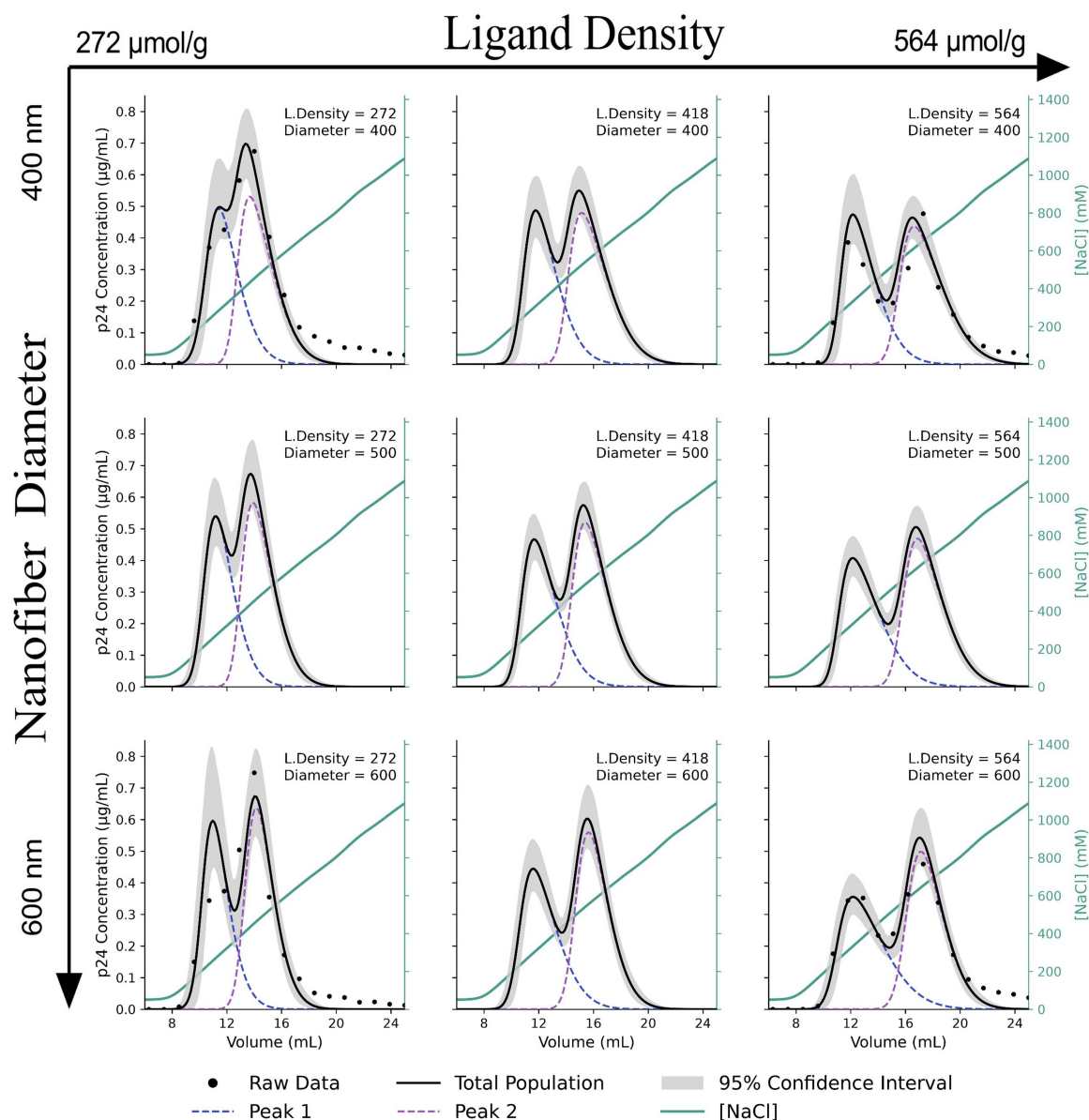


FIGURE 8 | Simulated LV Q-nanofiber gradient elution profiles at increasing nanofiber diameter and ligand density for a fixed porosity (0.6) and contact time (3 min). Profiles were generated by obtaining skewed-Gaussian model parameters from the initial fitting of raw data. MLR equations were then generated for each of the 8 individual skewed-Gaussian model parameters across the two populations. These equations enable calculation of skewed-Gaussian parameters for all DoE factor settings, including those not experimentally tested. The resulting parameter sets were used to simulate Peak 1, Peak 2, and Total Population elution profiles for each ligand density and nanofiber diameter combination, allowing visualization of how incremental increases in these factors influence the overall elution profile shape. 95% confidence bands represent the uncertainty in the fitted MLR equation coefficients propagated to the simulated elution profiles via residuals bootstrapping (see Section 2.8).

remaining largely unchanged. As hypothesized, this Peak 2 shift is consistent with adsorbent modifications that are thought to enhance multipoint binding (Figure 1A).

Because Peak 2 binding is thought to be mediated by highly charged, long-chain GAGs, this population may be more sensitive to adsorbent features that affect multipoint interaction. In contrast, changes in nanofiber design appear to have only minor effects on Peak 1 retention. Although increasing ligand density raised Peak 1 elution [NaCl] by 49 mM, no other factors were identified. While altered mass-transfer or flow dynamics could theoretically contribute to the peak shifts, previous work with

these fibers showed that more open pore structures reduce mass-transfer resistance (Ovari et al. 2024). If mass transfer were the dominant mechanism, these changes should also affect both LV peaks similarly, which is inconsistent with the population-specific behavior observed here. Overall, the simulations indicate that increased peak separation is driven primarily by an increase in [NaCl] required to elute the stronger-binding Peak 2 LV subpopulation. This differential sensitivity to nanofiber design is advantageous as it improves the potential to isolate Peak 2 from Peak 1. Although not explored here, further modest increases in ligand density or fiber diameter may yield additional gains in separation.

The results from this modeling methodology show that the Q-nanofiber structure capable of maximum peak separation and recovery is identified as (porosity = 0.6, ligand density = 564 $\mu\text{mol/g}$, diameter = 600 nm). One caveat to this selection is that increasing fiber diameter at higher ligand densities produced a modest increase in Peak 1 spreading (defined by s_1) (Figure 8). This could be considered undesirable as it leads to elution of more Peak 1 LV within the Peak 2 region. However, if VSV-G vesicles eluted in peak 1 are less sensitive to this adsorbent change and do not undergo similar tailing, it could increase the intra peak 1 selectivity of LV from VSV-G vesicles, leading to a better separation.

3.8 | Validation of Optimal Q-Nanofiber Structure for Isolation of the Peak 2 LV Subpopulation

The modeling results identified an adsorbent design of (porosity = 0.6, ligand density = 564 $\mu\text{mol/g}$, and diameter = 600 nm). This structure predicted a maximum total particle recovery of 73.6% and Peak Separation of 306 ΔmM NaCl. However, these are only model-predicted values, and the functional particle recovery of these adsorbents was yet to be determined. For the purposes of Q-nanofiber optimization, it was also assumed that Peak 1 LV co-eluted with VSV-G vesicles in this region (Figures 2

and 3) as VSV-G concentration could not be measured for all runs. This assumption must also be experimentally verified. The chosen nanofiber design was therefore validated to quantify both functional LV recovery and its ability to isolate Peak 2 LV from the major VSV-G signal, as quantified by western blot band intensity.

For validation experiments, 150 CV of CCH was loaded onto the Q-Nanofibers at 50 CV/min in triplicate. Although higher loads and flowrates could have been used, these conditions reflect those practically achievable in large-scale manufacturing whilst maintaining a contact time $\approx$ 3 min. Figure 9A,B show highly consistent elution profiles for both p24 and functional titer. The minimal variability across the three replicates indicates robust purification performance with these adsorbents. Peak Separation values also closely matched model-predicted values (288 ΔmM NaCl), with total particle recovery slightly exceeding the model prediction ($86 \pm 3\%$ p24), likely due to the higher load challenge, which was previously associated with reduced time-dependent loss (Pamenter et al. 2023). Crucially, high functional LV recoveries ($64 \pm 10\%$ TU) were confirmed.

Figure 9C shows the normalized VSV-G western blot band intensity profile overlaid with p24. A pronounced VSV-G intensity peak occurs in the Peak 1 LV region, demonstrating

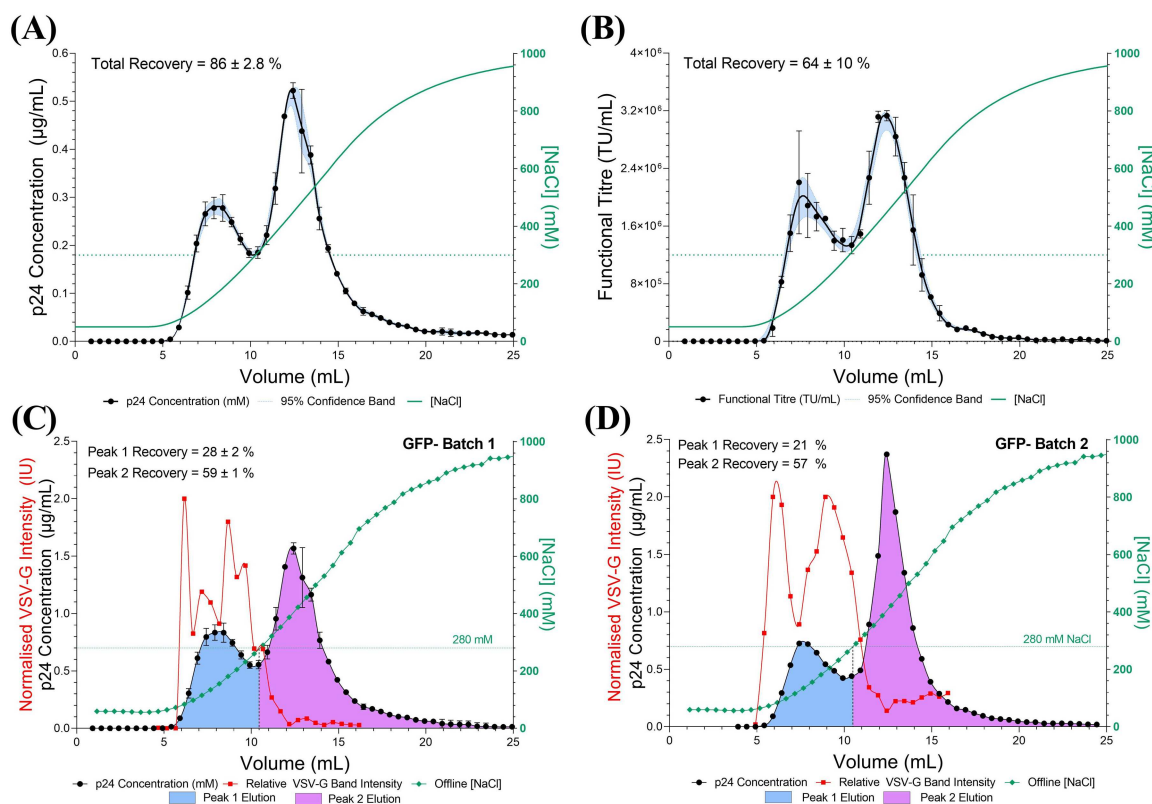


FIGURE 9 | Q-Nanofiber validation runs for gradient elution of two LV-GFP batches. Batch 1 corresponds to the material used for nanofiber optimization, and Batch 2 is the same material used in Figures 2 and 3 for the T-cell experiments. Error bars represent ± 1 SD for $N = 3$ biological replicates unless otherwise stated. (A) p24 gradient elution profile for GFP Batch 1. A smoothing spline was applied to the data, with its 95% confidence band indicated. Average cumulative recovery is given on figure. (B) Functional titer gradient elution profile for GFP Batch 1, with a smoothing spline and associated 95% confidence band shown. Average cumulative recovery is given in the figure. (C) Elution profile of normalized VSV-G intensity for GFP Batch 1, with the p24 elution profile plotted again for reference. VSV-G intensity was semi-quantitatively measured from Western blot band intensity. All samples were diluted to 0.03 $\mu\text{g/mL}$ p24. Therefore, the signal represents VSV-G per unit p24. (D) Elution profiles of p24 concentration and normalized VSV-G intensity for GFP Batch 2.

that on a per-particle basis, Peak 1 contains over an order of magnitude more VSV-G than Peak 2. As functional and total LV particle counts are broadly similar between the two peaks, this elevated VSV-G signal most likely reflects the presence of VSV-G-containing vesicles co-eluting with Peak 1 LV. It is noted that this measure provides only a semi-quantitative indication of VSV-G vesicle presence, and more comprehensive techniques for quantifying VSV-G and other vesicles during LV AIEX have recently been reported (Kocot et al. 2026). However, these were not available to us at the time of the experiment. Nonetheless, Figure 9C confirms that the chosen nanofiber design effectively isolates Peak 2 LV from most of the VSV-G signal. To confirm this performance was consistent across LV batches, a second LV-GFP batch was generated and purified using the chosen Q-nanofiber (Figure 9D). Again, isolation of Peak 2 LV from the main VSV-G signal was achieved.

Notably, the high separation capacity of these nanofibers was achieved whilst also attaining high functional and total LV particle recoveries. Furthermore, the overall [NaCl] required for elution was low ($< \sim 700$ mM NaCl). Figure 9C,D indicates a hypothetical wash for removing VSV-G particulates (280 mM NaCl). Despite losing some Peak 1 LV in this wash, overall process recoveries of $\sim 60\%$ are still expected when collecting Peak 2 only. This recovery is substantially higher (~ 2 – 3 fold) than typical large-scale membrane-based AIEX processes. The chosen Q-nanofiber therefore presents a promising next-generation adsorbent for purification of LVs where separation capacity is key.

4 | Conclusion

These results demonstrate that LV subpopulations eluted at different points in the AIEX gradient elution profile differ markedly in their T-cell transduction efficiency and cytotoxicity. LV eluted in Peak 1 increased %CD3⁺GFP⁺ cells at low doses. However, this was accompanied by an early plateau followed by a subsequent decline in %CD3⁺GFP. Peak 1 also exhibited a sustained drop in viability (Figure 2). In contrast, Peak 2 saw no viability loss or plateau. Peak 1's inhibitory effect on transduction efficiency was attributed to (+)VSV-G vesicles, which were shown to reduce the %CD3⁺GFP⁺ T cells in a dose-dependent manner (Figure 3D). T-cell viability reductions were attributed to some vesicle impurity present only in Peak 1 (Figure 3E), as this material reduced viability regardless of whether VSV-G was present. However, the precise identity of this impurity, or impurities, remains unknown. As VSV-G inclusion exacerbated Peak 1 viability loss (Figure 3E), this could indicate a cytotoxic vesicle impurity whose T cell entry is enhanced by VSV-G. Further work is needed to better quantify particulate species present in these fractions, as they appear intrinsically linked to LV product potency.

As LV manufacturing process yields improve, and with the rise of in vivo CAR-T therapy, we predict that separation goals may shift from maximizing overall recovery to isolating efficient LV subpopulations. Considering this and our T-cell transduction findings, a Q-nanofiber adsorbent capable of isolating the Peak 2 LV subpopulation at high yield was designed. Using a functional DoE approach, the effect of nanofiber diameter, ligand

density, porosity and time-spent in the adsorbed state (contact time) on the LV gradient elution profiles were explored (Figure 5). LV loss with contact time was greater at lower porosity, reducing from 59% to 24% ($t = 3$ – 50 min) at porosity = 0.4, and from 73% to 37% ($t = 3$ – 50 min) at porosity = 0.6 (Figure 4). Surprisingly, we found no impact of ligand density on time-dependent LV loss. This could indicate that ligand distribution, determined by the adsorbents 3D structure, is the critical parameter influencing LV time-dependent loss mechanisms and not basic ionic-capacity. However, the range of explored ligand densities was small.

Separation of Peak 1 and Peak 2 LV subpopulations during gradient elution (Peak Separation) increased significantly with increasing ligand density and nanofiber diameter (141 – 306 ± 28 Δ mM NaCl) (Figure 7). Simulations of LV elution profiles (Figure 8) indicated that this increase occurs predominantly from an increase in the Peak 2 elution [NaCl] ($+217$ mM). Peak 1 was largely unaffected. These population-specific binding differences are perhaps expected. As we believe Peak 2 LVs bind via GAGs, they are likely more sensitive to adsorbent-design changes that increase multipoint attachment due to the long-chain multivalent nature of GAGs (Pamenter et al. 2024). This differential impact between LV subpopulations offers the potential to further refine adsorbent structure and AIEX conditions for greater selectivity. Recent work has applied arginine elutions for increased LV separability, and similar selectivity-enhancing strategies could be explored with these adsorbents (Kocot et al. 2026).

The models (Figures 4,8) in this work indicate that nanofiber structure can be tuned to increase peak separation without compromising LV recovery. Thus, the (porosity = 0.6, ligand density = 564 μ mol/g, diameter = 600 nm) nanofiber was further validated for its ability to isolate Peak 2 LV from VSV-G impurities. High functional LV recoveries (64% TU) were obtained across 3 validation runs (Figure 9A,B), with clear isolation of the Peak 2 LV subpopulation from the major VSV-G impurities confirmed for two LV batches (Figure 9C,D). Critically, this was achieved using low [NaCl] (< 700 mM). This work highlights the need to tailor AIEX adsorbents to the specific separation needs of LV and elucidate the interplay between each adsorbent classes structural design features (e.g., pore size distribution and ligand density in membrane systems) and LV chromatographic behavior. Reliance on retrofitted technologies cannot continue if the industry is to deliver purification tools suited to modern gene-therapy vector manufacturing.

More broadly, even with rationally engineered AIEX adsorbents, the high structural similarity between vesicle impurities (e.g., VSV-G vesicles) and LVs makes their separation difficult when relying solely on charge-based methods, resulting in the sacrifice of product for purity. This is evident in this work as the Peak 1 LV subpopulation would need to be removed alongside VSV-G vesicles (Figure 9C,D). However, this LV subpopulation displayed markedly higher transduction efficiency than Peak 2 at low doses and could be favorable in some applications (Figure 2). Completely eliminating—or better still preventing the formation of—Peak 1-associated vesicles whilst retaining Peak 1 LV could remove the transduction inhibitory effect, leaving behind an inherently more potent LV

subpopulation. This may require engineered cell cultures methods to favor production of efficient LV subpopulations whilst suppressing vesicle impurity formation, or orthogonal purification strategies where identification of envelope markers overexpressed on vesicle impurities could allow for targeted removal with affinity ligands.

The results in this work present preliminary steps towards understanding which particulate species impair LV therapeutic efficacy and how they may be removed. However, a significant body of future research is needed to utilize more advanced analytical techniques that quantify product-like impurities. Ultimately, these need to be correlated directly to the final in vivo drug potency. Such knowledge is essential for guiding future LV purification design, as without it, the goals of LV separation remain ambiguous.

Author Contributions

George Pamenter and Daniel G. Bracewell conceived the study. T-Cell and peak purification experiments were designed and executed by George Pamenter, Thomas Williams-Fegredo, and Anurag Kulkarni. Gyorgy Ovari and Farzad Foroutan optimized and executed nanofiber manufacture. George Pamenter, Gyorgy Ovari, Matthew Townsend, and Daniel Bracewell designed Nanofiber chromatography experiments. Nanofiber chromatography experiments were executed by George Pamenter and Gyorgy Ovari. George Pamenter and Stephen Goldrick developed the analysis and modeling methodology. Research was performed in the laboratory of Lee Davies and Kyriacos Mitrophanous. George Pamenter, Daniel G. Bracewell, Lee Davies, and Stephen Goldrick wrote the manuscript with contributions from all other authors.

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Conflicts of Interest

Daniel G. Bracewell, Stephen Goldrick, George Pamenter, and Gyorgy Ovari declare no conflict of interest. Anurag Kulkarni, Thomas Williams-Fegredo, Lee Davies, Carol Knevelman, and Kyriacos Mitrophanous were all employees of OXB at the time the research was conducted. None of the authors or their employer received any additional remuneration for this work. Matthew Townsend and Farzad Foroutan were employees of Cytiva at the time the research was conducted, but did not receive any additional remuneration for this work.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Figure S1: Scouting studies and representative chromatograms for Anion-Exchange (AIE) protocol. Individual runs are given. (A) Dynamic binding capacity (DBC) for a [porosity = 0.5, diameter = 500 nm, ligand density = 564 μmol/g] nanofiber. Load is given in column volumes (CVs) of bioreactor material pre-dilution. 50 CV (100 CV post-dilution) load point is indicated on figure. The total p24 concentration in the loaded 1:1 diluted CCH was 0.22 μg/mL. We have previously shown that 'free' soluble p24 protein does not bind to AIE membranes (Pamenter et al. 2023). The unchanging concentration (~0.02 μg/mL) observed throughout the experimental range therefore represents the baseline level of free p24 protein present in the CCH that does not bind to the column. This confirms that no LV breakthrough occurred across the tested loading range. (B) p24 gradient elution profile conducted at the end of the DBC.

Figure S2: (A) DoE experimental conditions and run number. (B) Flowthrough p24 mass of each DoE run. No trends were identified in flowthrough mass using MLR. Thus, all samples remained underloaded.

Figure S3: Principal component analysis (PCA) of eluted LV mass across the elution profile. For PCA, each elution profile was segmented into 75 mM NaCl fractions and integrated to quantify total LV mass eluted in each NaCl range (A). Applying PCA to these mass fractions—a full correlation matrix is provided in Sup.Figure 4—indicates that PC1 (46% variation) primarily captures variability of the high-salt elution fractions (500–825 mM), while PC2 (39.8% variation) reflects variability in the low-salt elution fractions (125–500 mM). (A) Example deconstruction of LV elution profiles for 564 μmol/g (i) and 272 μmol/g (ii) ligand densities. Linearly interpolated profiles were segmented at 50, 125, 200, 275, 350, 425, 500, 575, 650, 725, 800, 875, and > 875 mM

NaCl. Integration of the interpolated profile between these cut-points was used to calculate the LV mass eluted within each segment. (B) PCA scores plot for PC1 and PC2. (C) Loadings plot for PC1 and PC2, with low-NaCl (green) and high-NaCl (purple) elution segments indicated.

Figure S4: Full correlation matrix for integrated peak areas in each salt segment. Densities are displayed individually. Bottom-left of the matrix gives linear correlation plots with 272 $\mu\text{mol/g}$ (Blue) and 564 $\mu\text{mol/g}$ (Red) Ligand Densities indicated separately. Top-right gives corresponding correlation values and significance circles.