Physical and biological interactions of protein therapeutics at the injection site are key determinants of subcutaneous bioavailability (Ryman and Meibohm, 2017; Ovacik and Lin, 2018). Reduced absorption due to pre-systemic drug clearance from the subcutaneous space may result from a variety of factors, including local catabolism at the site of injection, target-specific binding prior to vascular entry, or variable non-specific interactions with extracellular matrix (ECM) components leading to protein retention, e.g., charge-dependent binding (Mach et al., 2011; Richter et al., 2012). Critically, questions regarding the relative contribution of hypodermal vasculature as a primary route of subcutaneous absorption of protein therapeutics remain unanswered. For example, large (> 150 kDa) proteins like monoclonal antibodies (mAbs) have been reported to be preferentially absorbed into the lymphatic circulation, but smaller protein therapeutics (4-60 kDa) administered at sufficiently high doses may directly traverse the blood endothelium to a significant degree (McLennan et al., 2005; Richter et al., 2012; Kagan, 2014; Thomas and Balthasar, 2019). New experimental approaches are

1 Introduction

Subcutaneous delivery is now generally favored as the route of administration for protein therapeutics due to improved patient access through self-administration, lower clinical costs, and the option for extended-release formulations (McLennan et al., 2005). Yet, the development of subcutaneously delivered protein therapeutics has been hindered by a poor understanding of the factors affecting subcutaneous bioavailability, typically assessed systemically by the dose-normalized ratio of total serum exposure following a subcutaneous dose relative to that obtained after intravenous administration (Sánchez-Félix et al., 2020). Preclinical animal models are often poor predictors of drug absorption behavior in humans due to interspecies differences (Richter et al., 2012), which can result in drug development delays and early clinical stage candidate attrition. Thus, the generation of methods to predict subcutaneous bioavailability in humans with high fidelity would represent a critical advancement for the preclinical development of protein therapeutics.

Microphysiological Endothelial Models to Characterize Subcutaneous Drug Absorption

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Abstract

The high variability in subcutaneous bioavailability of protein therapeutics is poorly understood, contributing to critical delays in patient access to new therapies. Preclinical animal and in vitro models fail to provide a physiologically relevant testbed to parse potential contributors to human bioavailability, therefore new strategies are necessary. Here, we present a microphysiological model of the human hypodermal vasculature at the injection site to study the interactions of administered protein therapeutics within the microenvironment that influence subcutaneous bioavailability. Our model combines human dermal endothelial cells, fibroblasts, and adipocytes, self-assembled into three-dimensional, perfusable microvessels that express relevant extracellular matrix. We demonstrate the utility of the model for measurement of biophysical parameters within the hypodermal microenvironment that putatively impact protein kinetics and distribution at the injection site. We propose that microphysiological models of the subcutaneous space have applications in preclinical development of protein therapeutics intended for subcutaneous administration with optimal bioavailability.
needed to directly evaluate the contributions of these and other injection site-specific dosing strategies to overall pharmacokinetic behaviors.

In the absence of reliable preclinical in vivo animal data, alternative strategies have emerged to address factors affecting subcutaneous bioavailability, yet most remain limited in their effectiveness. For example, in silico models have yet to consistently predict bioavailability through mechanistically or empirically-driven simulations (Kagan, 2014). Ex vivo tissue models such as Hyposkin have been reported that incorporate the dermal, epidermal, and subcutaneous layers, which may provide important estimates of drug biodistribution within subcutaneous tissue (Na et al., 2022). However, the current lack of perfusable lymphatic and vascular systems hinders our ability to study routes of systemic entry of therapeutic proteins. Several published in vitro models of therapeutic protein absorption exist, but most focus purely on drug physicochemical properties as determinants of subcutaneous absorption (e.g., solubility, aggregation) without addressing the requisite 3D cellular architectural context of the subcutaneous microenvironment as barriers to absorption (Bown et al., 2018). Dermal models that do contain human cells have reached increasing levels of complexity, including microphysiological systems (MPS) harboring multiple dermal cell types in three-dimensional (3D) morphologies (Sutterby et al., 2020; Risueño et al., 2021). However, those existing MPS models do not provide the capability to measure critical parameters governing protein vascular or lymphatic distribution but instead focus on the dermal epithelium for transdermal drug absorption or disease studies.

Here, we attempt to bridge this knowledge gap by presenting a new microphysiological model representing the human subcutaneous space that allows the direct quantitation of parameters that influence bioavailability, including drug transport across vascular/lymphatic endothelium and drug diffusional characteristics within a representative ECM.

2 Methods

Formation of dMVNs in microfluidic devices

All cellular components of the dMVNs were initially tested for mycoplasma by the supplier and re-tested in our laboratory every 6 months. Primary dermal microvascular endothelial cells (EC, Lifeline, #FC-0042) and primary dermal fibroblasts (FB, Lifeline, #FC-0024) were cultured to passage 5 in Vasculife Endothelial Medium (Lifeline, #LL-0003) or Fibrolife Fibroblast Medium (Lifeline, #LL-0011), respectively, both changed every 2 days. Adipose-derived stem cells (ADSC, Lonza, # PT-5006) were initially cultured to passage 3 in ADSC Growth Medium (Lonza, #PT-4505, changed every 2 days), switched upon cell confluence to Subcutaneous Preadipocyte Differentiation Medium (Zenbio, #DM-2), which was changed every 4 days up to day 12 to differentiate the cells to adipocytes (AdC). The cell mix was seeded in the central channel of AIM microfluidic devices (Sigma Aldrich, # DAX01-1PAK) at a concentration of 6:1:1x10^6 cells/mL (EC:FB:AdC) in a fibrin gel as described elsewhere (Offeddu et al., 2021a; Hajal et al., 2021). Specifically, cells were separately suspended in a thrombin solution (4 U/mL, Sigma, #T4648) in Vasculife medium at 6X their final concentrations, i.e., 36:6:6x10^6 cells/mL (EC:FB:AdC). The suspensions were combined in equal volumes, and a total of 6 µL of combined cell suspension was mixed with 6 µL of fibrinogen solution (6 mg/mL, Sigma #F8630) in PBS. The mix was quickly injected into three AIM microfluidic devices, where it gelled within 2-3 min, and the procedure was repeated for the required number of chips for each experiment. The dMVNs self-assembled over 5-7 days with daily Vasculife Endothelial Medium changes through the side channels of the microfluidic device, whereby the cells within the 3D fibrin matrix rearranged to form perfusable interconnected vascular microvessels bridging the side channels (Fig. S1). While it is expected that the daily medium changes introduced small transient fluid flow across the gel and dMVNs, driven by pressure differences not larger than 50 Pa, these would have equilibrated within minutes (Offeddu et al., 2019b). The dMVN models formed were thus used under static conditions on day 7.

Imaging of the dMVNs was performed on an Olympus FV-1200 confocal microscope, staining vascular ECs with a CD31 monoclonal antibody (1:200, Abcam, #ab32457) or Ulex Europaeus Agglutinin I (UEA I, 1:200, Vector Laboratories, #DL-1068), AdCs with BODIPY (lipid vacuoles, ThermoFisher, #D3922), and using 4’,6-diamidino-2-phenylindole (DAPI, ThermoFisher, #D1306) and phalloidin (ThermoFisher, #A2287) to visualize the cell nuclei and cytoskeletons, respectively. The ECM components of the dMVNs were stained with a monoclonal antibody for HS (Amsbio, #370255) and a binding protein for HA (Sigma Aldrich, #385911), as done previously (Offeddu et al., 2021a). All reagents of animal origin used were chosen due to established practices in our laboratory, which will strive to find synthetic alternatives in the future. Alternative synthetic matrices are already being explored in our laboratory to form MNV models, but a better candidate than fibrin has not been found yet.

Fluorescein isothiocyanate (FITC) labeling of proteins

Cytochrome C from bovine heart (Sigma Aldrich, # C3131) and β2-microglobulin from human urine (Prospec, #PRO-553) were incubated with NHS-FITC (ThermoFisher, #46410) at a 10:1 molar excess in freshly prepared reaction buffer (0.1 M sodium bicarbonate, 0.1 M sodium carbonate, pH 9.0) for 60 min at room temperature. The reaction volume was 1 mL, and the protein concentration was 1 mg/mL. Afterwards, the samples were individually dialyzed against a total of 3 L each of 1X PBS, pH 7.4 over 24 h at 4°C in Slide-A-Lyzer™ dialysis cassettes with a 7 kDa molecular weight cutoff membrane (ThermoFisher, #66370). Total protein concentration was measured via the Bradford method using the Bio-Rad protein assay dye reagent (Bio-Rad, #5000006). Labeled proteins were stored at 4°C until use.
Monoclonal antibodies were incubated with NHS-Fluorescein (ThermoFisher, #46409) at a 5:1 molar excess in freshly prepared reaction buffer (0.1 M sodium bicarbonate, pH 8) for 60 min at room temperature. The reaction volume was 0.4 mL, and the protein concentration was 6 mg/mL. Afterwards, samples were desalted into 100 mM phosphate-buffered saline (pH 7.4) using 40 MWCO Zeba spin desalting columns (Thermo Scientific, #87766).

**Measurement of basolateral-to-apical protein permeability**

Assessment of endothelial permeability was carried out with solutes conjugated with FITC, as described above (cytochrome C, β2-microglobulin), or purchased pre-labeled: dextran (70 kDa, Sigma Aldrich, #FD70), serum IgG (Sigma Aldrich, #F9636), transferrin (Rockland, #009-0234), albumin (Abcam, #ab8030), IgG Fab fragment (Jackson Laboratories, #115-097-003), insulin (NanoCS, #IS1-FC-1), and ovalbumin (ThermoFisher, #O23020). The matrix of the dMVNs was permeated with the solutes by replacing medium in the side channels of the devices with Vasculife Endothelial Medium containing 0.1 mg/mL of solute, incubated for 30 min. The medium was then again replaced with fresh Vasculife Endothelial Medium, thus washing out the solute from the perfusable microvessels. Permeability was immediately measured as described previously (Offeddu et al., 2019b; Hajal et al., 2022). Briefly, confocal microscopy stacks were imaged on the confocal microscope above with a 10X objective, z-spacing of 5 µm and total stack thickness of 50 µm, at times 0 min and 9 min. Through confocal microscopy, the images show a fluorescent matrix with darker vessel spaces (where the solutes were washed out) progressively becoming brighter as the solutes cross the endothelium from the gel matrix into the microvessels (Fig. 3a).

Analysis of the images was performed with the software ImageJ, distribution FIJI (Schindelin et al., 2012), exactly as previously detailed (Offeddu et al., 2019b; Hajal et al., 2022), measuring the average fluorescence intensity (proxy for solute concentration) in the matrix and vessel spaces over time, thus yielding the morphological parameters of the dMVNs: volume fraction and specific surface area, and solute permeability. In this case, the permeability measured was from the basal side to the apical side of the endothelium, through measurement of the increase in fluorescence intensity within the vessel space. Average vessel diameter was calculated from knowledge of the vascular volume fraction and specific surface area in the volumes considered, assuming a cylindrical geometry of the vessels as done previously (Offeddu et al., 2019b).

**Measurement of protein-matrix interactions**

Diffusivity and immobile fraction of the solutes in the dMVN matrix was measured using fluorescence recovery after photo-bleaching (FRAP) as reported previously (Offeddu et al., 2019a). The matrix was permeated with each solute as above, a spot 30 µm in diameter was then bleached in regions adjacent to vessels, and images were recorded every 0.2 s. Analysis of the fluorescence recovery in the bleached spots was carried out using the *frap_analysis* plugin in the software MATLAB (MathWorks, version R2017b) yielding the diffusivity and immobile fraction of the solutes.

**Measurement of vascular morphology in human skin biopsies**

Fixed human skin biopsies in paraffin blocks were obtained from Genoskin Inc. for three patients (Patient 1: 33-year-old female, BMI 25.8, abdomen sample, Fitzpatrick skin type classification 3; patient 2: 39-year-old female, BMI 25.7, abdomen sample, Fitzpatrick skin type classification 2; patient 3: 45-year-old female, BMI 25.0, abdomen sample, Fitzpatrick skin type classification 2). The blocks were sectioned into 25 µm-thick slices. Following antigen retrieval, the slices were stained with the CD31 antibody detailed above, DAPI, and a monoclonal antibody for podoplanin (PDPN, Biolegend, #337003). Through hydration, the slices swelled to approximately double their thickness, and confocal microscopy stacks 50 µm in thickness could be imaged. Analysis of vascular volume fraction, specific surface area, and average vessel diameter was performed as done above for the dMVNs, for either blood (CD31<sup>high</sup>) or lymphatic (CD31<sup>low</sup>, PDPN<sup>high</sup>) vessels.

**Formation of lymphatic vascular model in microfluidic devices and solute drainage assay**

Dermal microvascular lymphatic models were generated based on our previous protocol (Serrano et al., 2022). Briefly, human dermal lymphatic endothelial cells (#CC-2543, HDLMEC, Lonza) were cultured in Vasculife Endothelial Medium supplemented with 6% FBS (Invitrogen) up to passage 6-8. A single-gel middle channel microfluidic device with two parallel, adjacent media channels was implemented based on previous designs from our lab (Farahat et al., 2012). After standard soft lithography preparation (Shin et al., 2012), fibrinogen from bovine plasma (Sigma) was dissolved to a concentration of 5 mg/mL and mixed over ice with thrombin (Sigma, 4 U/mL) at a ratio of 1:1. The resulting fibrin solution was injected into the middle gel channel and allowed to polymerize for 15 min. Subsequently, human plasma fibronectin (EMD Millipore) was diluted to a concentration of 100 µg/mL in PBS prior to being injected into one of the media channels where the lymphatic endothelial cells would be seeded and allowed to incubate for 30 min. Finally, lymphatic cells were resuspended to a concentration of 3 x 10<sup>6</sup> cells/mL, and 30 µL of this suspension was introduced to the fibronectin-coated channel. Devices with seeded cells were placed in the incubator and allowed to attach for a period of 24 h.

To grow 3D lymphatic vasculature in the gel channel, a pressure difference of ~100 Pa was established to guide flow towards the lymphatic endothelial monolayer. The resulting interstitial flow (~6 µm/s) initiated lymphatic sprouting against the direction of flow within the gel channel. The fluid pressure reservoirs were replenished on a daily basis to allow this constant flow during the course of 4 days. Using this on-chip lymphatics system, we measured the lymphatic drainage rate of the various proteins by introducing them in the opposite media channel, devoid of lymphatics. This time, a pressure difference of ~10 Pa was established, which imparts interstitial flow of ~1 µm/s towards the lymphatic channel, thus recapitulating physiological interstitial flow (~6 µm/s) towards the lymphatic channel.
flow guiding the proteins from the ECM compartment towards the lymphatics. As the proteins were absorbed by the lymphatics, lymphatic drainage was monitored via fluorescence correlation, by taking confocal stacks of 4 slices at 80 µm with a 10X objective during time intervals of 2-4 min. Analysis of the images was performed with the software ImageJ. From this data, we implemented a solute drainage rate estimation as done previously (Serrano et al., 2022), which allowed the quantification of the corresponding lymphatic drainage of each given protein.

Monoclonal antibody panel and measurement of physicochemical parameters

Nine mAbs were recombinantly produced in a consistent IgG1 isotype. This isotype, IgGz SEFL2.2, is glycosylated, with mutations to improve thermal stability via an engineered disulfide bond and to reduce binding to Fcγ receptors (Liu et al., 2017). The Fv domains were selected to exhibit a broad range of physicochemical behaviors without consideration of target biology. The mAbs were expressed in stable Chinese hamster ovary cells, followed by protein A affinity capture, cation exchange chromatography, and formulation into buffer (10 mM sodium acetate, 9% sucrose, pH 5.2) at a purity of > 95% by analytical size exclusion chromatography.

Several assays probing mAb physicochemical properties that may be useful for mAb in vivo distribution prediction have previously been described in the literature, including self-interaction propensity (Liu et al., 2014; Kelly et al., 2015; Bettis et al., 2018; Jones et al., 2019) (AC-SINS, Tagg, viscosity), charge (zeta potential) (Boswell et al., 2010; Bumbaca et al., 2012; Li et al., 2014; Datta-Mannan et al., 2015a,b; Schoch et al., 2015; Yadav et al., 2015), cross-interaction to various substrates (Kelly et al., 2015; Dostalek et al., 2017; Avery et al., 2018) including those of positively charged (poly-D-lysine ELISA), negatively charged (heparin chromatography (Kraft et al., 2020), PEI ELISA), hydrophobic (membrane preparation ELISA, hydrophobic interaction chromatography) heparin chromatography, PEI ELISA), or mixed (BVP ELISA (Hötzel et al., 2012)) character. These assays were implemented, as well as measurements of thermal stability (Tm), colloidal stability (ammonium sulfate precipitation and PEG precipitation), and non-specific protease stability (thermolysin). We also measured FcRn binding using FcRn chromatography (Schlothauer et al., 2013) to assess the impact of diverse Fab sequences on FcRn engagement (Schoch et al., 2015; Kelly et al., 2016). Details of these methods are included in the supplementary file 1.

Data interpretation and statistics

Single data points for each biological repeat are plotted in the graphs when possible without compromising clarity in the main text figures. Otherwise, single data points are provided in supplementary figures 1. For dMVNs samples, each repeat represents one device, three measurements per device. For human skin biopsy measurements, each repeat represents one patient, three measurements per patient. Statistical analysis of the data was performed with the software Prism (GraphPad, version 9), and the specific statistical test used is reported in each figure caption. Mean differences with p < 0.05 were taken as significant. Use of parametric tests was conducted following confirmation of the normal distribution of the data.

3 Results

3.1 Self-assembly of human hypodermal-like microvasculature in microfluidic devices

We applied an established methodology to assess drug permeability developed in our laboratory to construct vascular models containing dermal microvascular networks (dMVNs) within microfluidic devices (Chen et al., 2013; Whisler et al., 2013). Primary human dermal microvascular endothelial cells (ECs) and primary dermal fibroblasts (FBs) were co-cultured with human adipose stem cell-derived adipocytes (AdCs) in a 3D gel matrix. Over 5 days, the cells self-assembled into a microvasculature with perfusable lumens, where the stromal FBs and AdCs populated the gel matrix between microvessels (Fig. 1a, Fig. S1). Initial optimization of the model revealed that increasing numbers of FBs resulted in microvessels with altered diameters (Fig. S2a). Inclusion of stem-cell derived AdCs in the model also significantly impacted the morphology of the dMVNs (Fig S2b), with lower vascular density (22% compared to 42% without AdCs, by gel matrix volume) and microvessel average diameter (16 µm compared to 28 µm) but similar specific surface area of the endothelium (approximately 0.06 µm⁻¹).

Comparison of the dMVNs to human native dermal microvasculature is critical to determine the level of model fidelity. Therefore, we obtained fixed human skin biopsies and performed morphological analysis of blood microvessels in the three major layers of the skin containing blood vasculature: sub-epidermis, dermis, and hypodermis (subcutaneous). We utilized immunofluorescent staining of CD31 (PECAM1) and podoplanin (PDPN) to delineate vascular and lymphatic endothelial vessels, respectively (Baluk and McDonald, 2008) (Fig. 1b, Fig S2c). By comparison, the dMVNs exhibited higher vascular density than native dermal microvasculature (in the range of 5% by tissue volume), larger average diameter (approximately 5 µm), and similar specific surface area to the sub-epidermis and hypodermis microvasculatures, though higher than the dermis (Fig. 1c). Brightfield imaging confirmed the presence of latticed adipocytes within the subcutaneous layer (Fig. 1b, panel 3). Some collapse of vascular structures is expected in fixed biopsies (Imayama and Urabe, 1986), which may have resulted in the lower apparent vascular density and microvessel diameter in fixed samples when compared to dMVNs. Nonetheless, these results show that the dMVNs can, to a first approximation, recapitulate the native surface area of the endothelium per volume of tissue, a key parameter that determines the potential for molecular exchange of solutes between the interstitium and blood (Hu and Weinbaum, 1999).

3.2 Measurement of basolateral-to-apical endothelial permeability of proteins and their matrix interactions

Protein therapeutics injected subcutaneously have been shown to enter the blood through direct basolateral-to-apical trans-
without AdCs present. We observed an increase in dMVN permeability by a factor of approximately 3 with the addition of adipocytes, suggesting AdCs modify the microvessel phenotype in the dMVN systems (Fig. 2c).

We previously observed saturable apical-to-basolateral transcytosis of human serum IgG across the MVN endothelium, suggesting an active, or receptor-mediated, transport pathway (Offeddu et al., 2019b). To examine if a similar process occurred in the opposite direction, dMVNs were exposed to varying concentrations of human serum IgG. As done previously, we opted for a pooled preparation of serum IgG as representative of one of the most abundant proteins in blood without bias towards IgG subclass-specific transport (e.g., IgG1). Additionally, the use of serum IgG provided insight into the vascular uptake potential of protein across the blood endothelium (Sánchez-Félix et al., 2020). However, past work has demonstrated the fraction directly absorbed into the blood can vary with protein size (McLennan et al., 2005). To measure EC permeability of fluorescently labelled proteins, we adapted our previously developed methodology for apical-to-basolateral measurements in MVNs (Offeddu et al., 2019b; Hajal et al., 2022). Here, proteins were administered into the gel matrix of dMVN devices to represent the interstitial drug depot to enable monitoring of subsequent events leading to vascular transport (Fig. 2a). Protein permeability in dMVNs decreased with increasing molecular weight, approximating a plateau for molecular weights higher than approximately 60 kDa (Fig. 2b, Fig. S3a). Additionally, we examined the impact of AdCs on human serum IgG permeability in dMVNs with or without AdCs present. We observed an increase in dMVN permeability by a factor of approximately 3 with the addition of adipocytes, suggesting AdCs modify the microvessel phenotype in the dMVN systems (Fig. 2c).
general, the diffusivity of proteins in the gel matrix decreased with increasing molecular size (Fig. 3b, Fig. S3b1), where the data could be described by a power law that exhibited a diffusion-molecular size dependence (Fig. S41) within range of that previously reported in tissue (Clauss and Jain, 1990). Interestingly, serum IgG diffusivity also decreased with increasing protein concentration, from approximately 77 µm²/s at 0.1 mg/mL to approximately 39 µm²/s at 10 mg/mL (Fig. 3c), which may be explained by molecular crowding in the gel matrix pores (Sanabria et al., 2007).

An assessment of pseudo-irreversible protein binding to components of the gel matrix was enabled through quantification of the resultant immobile fraction (see Methods section), but this analysis did not reveal a clear trend with molecular weight (Fig. 3d, Fig. S3c1). However, we observed a decrease in permeability to a non-zero plateau above 60 kDa suggests a predominant role of transcytosis in the transport of proteins at or above the size of serum albumin.

Our dMVN device enables the quantification of parameters related to the interaction of protein analytes with components of the gel matrix, including the initial fibrin hydrogel and emergent cell-deposited matrix (i.e., the glycocalyx), putatively a determinant of molecular behavior within the dermal microenvironment. To this end, we employed fluorescence recovery after photobleaching (FRAP) to measure analyte diffusion post administration of fluorescently-labeled proteins permeated through the gel matrix of the devices (Fig. 3a), as was demonstrated previously in analogous MVN models (Offeddu et al., 2019a). In general, the diffusivity of proteins in the gel matrix decreased with increasing molecular size (Fig. 3b, Fig. S3b1), where the data could be described by a power law that exhibited a diffusion-molecular size dependence (Fig. S41) within range of that previously reported in tissue (Clauss and Jain, 1990). Interestingly, serum IgG diffusivity also decreased with increasing protein concentration, from approximately 77 µm²/s at 0.1 mg/mL to approximately 39 µm²/s at 10 mg/mL (Fig. 3c), which may be explained by molecular crowding in the gel matrix pores (Sanabria et al., 2007).

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as model probes with molecular weight equivalent to IgG – in conjunction with HA or HS degradation by hyaluronidase or heparinase, respectively (Offeddu et al., 2021a).

Basolateral-to-apical permeability across the dMVNs was similar for the differently charged dextrans (Fig. 4b), contrary to what we observed previously for apical-to-basolateral permeability in other MVNs. Positive-ly-charged molecules exhibited higher MVN permeability than neutral or negatively-charged molecules of the same molecular weight due to interactions with the negatively-charged EC glyco-calyx (Offeddu et al., 2019b). Here, we first confirmed through immunostaining that the ECs comprising dMVNs also express proteins associated with the glyco-calyx, namely hyaluronic acid (HA) and heparan sulfate (HS) (Fig. 4a). We then measured the transport parameters described above for neutral, negatively-charged, and positively-charged 150 kDa dextrans – used here as model probes with molecular weight equivalent to IgG – in conjunction with HA or HS degradation by hyaluronidase or heparinase, respectively (Offeddu et al., 2021a).

Basolateral-to-apical permeability across the dMVNs was similar for the differently charged dextrans (Fig. 4b), contrary to what we observed previously for apical-to-basolateral permeability in other MVNs (Offeddu et al., 2019b), although positively-charged dextran accumulation was observed throughout the dMVN device (Fig. S5). For the neutral and negatively-charged dextran, degradation of HS (and possibly HA, although not statistically significant) produced an increase in permeability consistent with the known role of the glyco-calyx as a molecular filter (Hu and Weinbaum, 1999; Tarbell and Cancel, 2016) and what was previously observed in other MVN models (Offeddu et al., 2021a).

Charge also affects interactions of the molecules with the gel matrix. Diffusivity for the positively-charged dextran was lower when compared to the neutral and negatively-charged counterparts (by a factor of approximately 0.5, Fig. 4c). Degradation of
Previously developed a separate MPS model of the dermal lymphatic microvasculature consisting of dead-ended vascular trees self-assembled by lymphatic ECs that sprout into a gel matrix (Serrano et al., 2022) (Fig. 5a). The morphology of the vessels in that model was compared to that of the tissue lymphatic microvasculature within fixed skin biopsies (Fig. 1b), which revealed an apparent lower specific surface area in the model (Fig. 5b). Similar to our blood microvascular measurements, lymphatic vessel collapse following sample fixation and preparation may have resulted in errors in our analysis (Imayama and Urabe, 1986).

The MPS lymphatic model enables the measurement of transport across the lymphatic endothelium under physiologic levels of interstitial flow (Serrano et al., 2022). This methodology can yield the solute drainage rate for a protein of interest, which more closely resembles values for large proteins observed in vivo. To compare lymphatic and blood endothelial transport, we first measured the solute drainage rate for serum IgG in the lymphatic model, finding no trend with increasing concentration (Fig. 5c). These findings contrasted with our observations in the blood microvasculature.

HA and HS did not affect the diffusivity of the neutral and negatively-charged dextrans but substantially increased diffusivity for the positively-charged dextran, from approximately 15 \( \mu \text{m}^2/\text{s} \) to 46 \( \mu \text{m}^2/\text{s} \) for HA and HS treatment, respectively. This behavior may be expected due to attractive interactions between differently charged species (Li et al., 2014; Xu and Esko, 2014). Indeed, the immobile fraction of positively-charged dextran was higher than that of its counterparts (Fig. 4d) and was significantly decreased after HS degradation, from 0.48 to 0.29. At the same time, the immobile fraction of negatively-charged dextran was also significantly lower than neutral dextran (0.06 compared to 0.18). These results further confirm that molecular charge plays a role in determining interactions with the ECM.

### 3.4 Assessment of lymphatic transport in an established microphysiological model

Protein therapeutic absorption from the subcutaneous injection site can also occur via entry across the lymphatic endothelium (McLennan et al., 2005; Sánchez-Félix et al., 2020). We previously developed a separate MPS model of the dermal lymphatic microvasculature consisting of dead-ended vascular trees self-assembled by lymphatic ECs that sprout into a gel matrix (Serrano et al., 2022) (Fig. 5a). The morphology of the vessels in that model was compared to that of the tissue lymphatic microvasculature within fixed skin biopsies (Fig. 1b), which revealed an apparent lower specific surface area in the model (Fig. 5b). Similar to our blood microvascular measurements, lymphatic vessel collapse following sample fixation and preparation may have resulted in errors in our analysis (Imayama and Urabe, 1986).

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3.5 Utility of the two endothelial models to differentiate amongst monoclonal antibodies and correlate with experimentally measured physicochemical properties

A panel of nine mAbs sharing a consistent human aglycosylated IgG1-derived structure (IgG1 SEFL) lacking effector function (Liu et al., 2017) and with diverse Fv domains was evaluated in the two microphysiological endothelial models (Fig. 6a). The obtained measurements were comparable to the serum IgG evaluated in Figures 2, 3 and 5. The dynamic range across the panel varied by 41.1-fold, 1.7-fold, 2.9-fold, and 3.7-fold for dMVN permeability, immobile fraction, diffusivity, and lymphatic solute drainage rate, respectively. In comparing binary relationships between each of the model parameters, only immobile fraction and solute drainage rate exhibited a Pearson correlation coefficient greater than |0.5| ($r = -0.53$; data not shown), suggesting that these parameters may be covariates.

To further compare serum IgG transport across the two endothelial network models, we translated the lymphatic solute drainage rate to an effective permeability by knowledge of the morphological lymphatic model parameters, vascular volume fraction, and specific surface area (Serrano et al., 2022) (Fig. 5d). Compared to the blood endothelium in the dMVNs, transport across the lymphatic endothelium in the lymphatic model is approximately two orders of magnitude higher for each IgG concentration tested. Furthermore, the difference increased with higher serum IgG concentrations due to saturation of blood microvascular transport. Overall, these results imply that for large proteins like serum IgG, lymphatic absorption dominates over direct blood absorption in the microphysiological endothelial models.

Fig. 5: Comparison to lymphatic microvascular model demonstrates preferential lymphatic absorption of large proteins
(a) Schematic diagram (left) and projected confocal microscopy image (right) of the lymphatic microvascular model described previously (Serrano et al., 2022). The scale bar is 200 µm. (b) Comparison of endothelial specific surface area between lymphatic microvascular model and skin layers in human biopsies, as shown in Figure 1b. (c) IgG drainage rate across lymphatic endothelial model as a function of protein concentration. (d) Normalized effective permeability of serum IgG across blood and lymphatic endothelial models with increasing protein concentration (left); n = 3. The schematic diagram (right) depicts preferential absorption of IgG in the lymph versus blood within the subcutaneous space. Significance assessed by one-way ANOVA; *, p < 0.05.
two dermal endothelial models, the antibodies were submitted to a battery of diverse protein attribute assays for hydrophobic interactions (hydrophobic interaction chromatography), FcRn binding (FcRn affinity), cross interactions (baculovirus particle binding, membrane preparation binding), charged interactions (poly-D-lysine binding, PEI binding, heparin binding), protease stability (thermolysin stability), colloidal stability (ammonium sulfate precipitation, PEG precipitation), and self-interaction (Tm, Tagg, zeta potential, AC-SINS, viscosity). A scatterplot matrix comparing the results of the physicochemical assays and the endothelial model parameters is shown in Figure S6\(^1\). Meaningful correlations were determined by the Pearson \(r\) value for each model parameter vs. each assay parameter and reported in Figure 6b for \(r\) greater than \(0.5\) and no single outliers driving the observed relationship (e.g., immobile fraction vs. baculovirus particle binding, membrane preparation binding, poly-D-lysine binding, PEI binding and viscosity). Only two of the five correlations that fit the above criteria had \(p\)-values < 0.05 (Immobilise fraction vs \(T_{agg}\) onset and diffusivity vs PEG precipitation).

4 Discussion

We have developed a new MPS model of the human hypodermal microvasculature that can be used to quantify parameters related to protein interstitial transport and vascular absorption. The dMVN model recapitulates key characteristics of the subcutaneous microenvironment, namely a perfusable 3D vascular architecture containing native dermal stromals. The average vessel size in the dMVNs (~16 µm, Fig. 1c) is only slightly larger than what has been reported in human skin, in the range of 10-12 µm (Braverman, 2000). The smaller vessel size measured here in patient biopsies (~5 µm) may potentially be due to the fixation process (Imayama and Urabe, 1986). Nevertheless, assuming a cylindrical vessel geometry, a decrease in vessel size by a factor of ~2 due to fixation would imply a decrease in vascular density in the fixed biopsies by a factor of 4, giving a value of ~16% \textit{in vivo} as opposed to the ~4% measured. This value compares well to the 22% vascular density measured in the dMVNs. Similarly, this approach gives a vessel specific surface area of \(2 \times 0.03 \mu m^2 = 0.06 \mu m^2 \textit{in vivo}\), which more closely matches the dMVNs (~0.06 \mu m\(^2\)). Therefore, the dMVNs system can successfully capture the morphology of the subcutaneous microvasculature \textit{in vivo}. Interestingly, the presence of the AdCs in the model contributes to this recapitulation, as without these cells the dMVNs show even larger vessel sizes (Fig. S2\(^2\)). Multi-cellular in vitro models have recently been applied to understand the crosstalk between AdCs and ECs (Hammel and Bellas, 2020). The AdCs included in the dMVNs do not appear to possess a fully mature phenotype, as observed by the lack of a single large lipid droplet, and improved methodologies are being developed to differentiate mature AdCs to

**Fig. 6:** Relationship between model output parameters and mAb physicochemical properties

(a) Permeability, immobile fraction, diffusivity, and drainage rate for a panel of 9 mAbs. (b) Table summarizing Pearson correlations and corresponding \(p\) values where \(r = < -0.5 \text{ or } > 0.5\) and correlation is not driven by a single outlier.

<table>
<thead>
<tr>
<th>Model parameter</th>
<th>In vitro assay parameter</th>
<th>Assay readout</th>
<th>Correlation co-efficient (Pearson (r))</th>
<th>(P) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immobile fraction</td>
<td>(T_{agg}) onset</td>
<td>Stability</td>
<td>0.73</td>
<td>0.02</td>
</tr>
<tr>
<td>Immobile fraction</td>
<td>Heparin binding</td>
<td>Positive charge patches</td>
<td>-0.58</td>
<td>0.10</td>
</tr>
<tr>
<td>Diffusivity</td>
<td>PEG precipitation</td>
<td>Stability</td>
<td>-0.71</td>
<td>0.03</td>
</tr>
<tr>
<td>Drainage rate</td>
<td>FcRn affinity</td>
<td>FcRn-mediated transit</td>
<td>-0.54</td>
<td>0.14</td>
</tr>
<tr>
<td>Drainage rate</td>
<td>PEG precipitation</td>
<td>Stability</td>
<td>-0.53</td>
<td>0.15</td>
</tr>
</tbody>
</table>
include in MPS models (Yang et al., 2021). More studies will be needed to investigate the effect of these cells on vasculogenesis, including the changes in vascular permeability we observed here (Fig. 2c).

Additionally, our dMVN studies demonstrated decreased basolateral to luminal microvascular permeability as protein size increased. This was anticipated because a non-fenestrated blood endothelial barrier will exhibit size-dependent permeability due to several factors that include the glycocalyx, surrounding ECM, and intercellular connections (Michel and Curry, 1999; Mehta and Malik, 2006). In general, microvascular permeability in continuous endothelial beds such as those found in skin occurs via paracellular and transcellular routes (Braverman, 2000; Mehta and Malik, 2006; Ono et al., 2017). Paracellular diffusion is restricted by adherens and tight junctions between adjacent endothelial cells (Mehta and Malik, 2006; Komarova and Malik, 2009). We have previously shown that MVN models possess physiological paracellular permeabilities due to the formation of these junctions in the 3D microvessels, resulting in values of the order of 10^8 cm/s, as also observed here for the dMVNs, compared to > 10^6 cm/s for the same molecule in endothelial monolayers on membrane inserts (Offeddu et al., 2019b). Due to the fixed size of endothelial junctions, past studies have established that microvascular permeability falls sharply with increasing molecular radius up to roughly the size of serum albumin (~60kDa) (Mehta and Malik, 2006; Polley, 2011; Corovic et al., 2015). The permeability of molecules in excess of this size threshold is less sensitive to further molecular weight increase that indicates a diffusion threshold (Mehta and Malik, 2006; Polley, 2011). Our results are consistent with this trend as dMVN permeabilities held relatively constant for proteins larger than albumin, attesting to the physiological relevance of the permeability results obtained in the dMVN model (Fig. 2c).

As molecular radius increases and paracellular diffusion rates across blood endothelial vessels decrease, the transcellular pathway predominates. Most macromolecules cross endothelial barriers via bulk/fluid-phase endocytosis and active transcytosis, though receptor-mediated endocytosis and transcytosis occurs for select proteins such as albumin (Tuma and Hubbard, 2003). Dermal MVN networks exhibited concentration-dependent permeability for serum IgG (Fig. 2d), indicating a saturable transport process that may be partly mediated by the neonatal Fc receptor (FcRn, FCGRT). FcRn is expressed throughout the adult human body and exists in a complex with β2-microglobulin (B2M). It binds to both albumin and IgG with high affinity at acidic pH. Endosomal FcRn binding promotes recycling or transcytosis of intact IgG. Vascular endothelial IgG internalization has been shown to partly be needed to investigate the effect of these cells on vasculogenesis, including the changes in vascular permeability we observed here (Fig. 2c).

In contrast to the size dependence observed in the microvasculature, protein entry into initial lymphatics is largely non-selective (Schmid-Schonbein, 1990; Porter and Charman, 2000; Swartz, 2001). Initial lymphatic endothelial cells lack a basement membrane and are focally anchored to the surrounding tissue. The cells are organized in oak-leaf arrangements with overlapping borders possessing intercellular breaks and sporadic junctions. Unlike the nanometer-sized junctions observed by electron microscopy in our blood microvascular models (Offeddu et al., 2019b), the gaps between lymphatic endothelial cells are expected to be several microns wide, permitting essentially unrestricted influx of interstitial components into the initial lymphatics (Schmid-Schonbein, 1990; Moore and Bertram, 2018; Breslin et al., 2019). Lymphatic drainage is largely dominated by local fluid and pressure dynamics that are heavily influenced by physical activity (Swartz, 2001; Breslin et al., 2019). This is consistent with the concentration-independent lymphatic drainage observed for serum IgGs (Fig. 5c).

Our normalized effective microvascular and lymphatic serum IgG permeabilities indicate significantly higher rates of lymphatic entry for each IgG concentration tested (Fig. 5d). These and our size-dependent dMVN permeability results agree with past subcutaneous dosing studies indicating the route of absorption is heavily reliant on the size of the administered protein (McLenman et al., 2005; Kagan, 2014). Previous work utilizing lymphatic cannulation models reported the percentage of the dose recovered within lymph increased with molecular size. At protein molecular weights of 30-40 kDa, most of the dose underwent lymphatic absorption (McLenman et al., 2005). In a separate study, non-invasive fluorescence imaging showed increased subcutaneous injection site residence time and draining lymph node exposure as protein size increased (Wu et al., 2012). Taken together with these and other past findings, the physiology of initial lymphatic uptake, and protein flux through the interstitial space, our permeability studies support the lymphatic network as the major route of absorption for monoclonal antibodies following subcutaneous administration (Schmid-Schonbein, 1990; McLennan et al., 2005; Breslin et al., 2019).

Protein diffusivity was a readily quantifiable parameter in our MPS model, and measurements obtained within the dMVN argue a degree of fidelity to that of living intact skin. The diffusivity values obtained in the current model for a diverse set of globular proteins (5.8-150 kDa) fell within range of previously published reports in animals (Clauss and Jain, 1990). Past diffusion analysis conducted within an experimental rabbit ear chamber reported that a power law (\(D = a(M_c)^b\)) best described diffusivity size dependence for linear FITC-dextran, noting a large discrepancy in fitted exponents between normal or neoplastic tissue (Nugent and Jain, 1984) (\(b = -2.96\) vs. \(-1.14\) for normal vs. tumor, respectively). Our diffusivity measurements within the dMVN device indicate a lesser dependence on molecular weight (\(b = -0.54\), Fig. S4). However, this discrepancy may be related to our focus on globular proteins rather than linear dextrans, where molecular shape can affect diffusivity so effective size (Rh) is likely the more meaningful parameter as noted by others (Clauss and Jain, 1990). To this end, when diffusivity was plotted as a function of hydrodynamic radius
(\(D = a(R_b)b\)), the size dependence fell within range \((b = -1.24)\) of that reported by Nugent and Jain (1984). It should be noted that the overall subcutaneous stromal recapitulation in the dMVN model remains limited due to the lack of large stromal cell populations like AdCs between microvessels. Yet, the results reported here for diffusivities measured in the vicinity of the microvessels, where the matrix was denser due to cellular deposition of additional bio-polymers (Fig. 4), show that the model can be used effectively to examine matrix effects in the vascular unit.

Biomolecular diffusivity is known to be highly sensitive to both tissue composition and density effects, where interstitial void volume, in particular, can vary dramatically depending on both tissue composition and density effects, where interstitial flow-mediated transport of molecules through the matrix (Offeddu et al., 2018). We observed a significant decrease in serum IgG diffusivity with the removal of AdCs, and we propose this to be an indirect effect due to both altered ECM composition and vascular morphology when AdCs are excluded during dMVN self-assembly and maturation. Specifically, the absence of AdCs resulted in both lower vascular density and vessel diameter, which together might increase molecular diffusion distances to the nearest vessel wall. Additionally, the ECM composition is likely altered by this extra cell type, where an interplay between key constituents (e.g., hyaluronic acid and other glycosaminoglycans, collagens, heparan sulfate) could affect interstitial void volume and charge density, such that an overall lower molecular resistance is established (De L. Davies et al., 2002; Thurber et al., 2008). Further work will be required to define the influence of AdCs on the dMVN microenvironment and protein therapeutic behavior during studies of subcutaneous absorption.

We confirmed the presence of both HA and HS as abundant constituents of dMVN glyocalyx. Both HA and HS are high in negative charge density (De L. Davies et al., 2002), and we were able to confirm ECM charge as a sensitive parameter governing molecular diffusion. As expected, positively charged DEAE-dextran exhibited the greatest sensitivity in both diffusion and immobile fraction to selective HA and HS depletion, highlighting the overall negatively charged nature of the ECM and phospholipids of cellular membranes. Notably, DEAE-dextran exhibited pronounced cellular and matrix retention apparent even under low magnification (Fig. S4). Our observations with charged dextrans in dMVNs suggest overall molecular charge (protein isoelectric point) to be a key physicochemical determinant of tissue disposition and clearance (Bumbaca et al., 2012).

Diffusivity of human serum IgG was found to decrease with increasing concentration, a phenomenon reported for measurements of green fluorescent protein variants in buffered solutions (Swaminathan et al., 1997), and extensively modeled for protein formulations in various hydrogels as due to molecular crowding, or “obstruction” (Amsden, 1999; Axpe et al., 2019). Additionally, an observed decrease in immobile fraction with increasing IgG concentration also supports saturable binding within the dMVN device. However, these observations with heterogenous serum IgG are likely non-specific, and characterization of expression of specific mAb target receptors within the dMVNs will be required to account for their potential influence on the range of diffusion and immobile fraction values measured across the panel of mAbs.

While not statistically significant with only nine mAbs (Pearson \(r = -0.54, p = 0.14\)), the apparent negative correlation between FcRn binding and lymphatic drainage rate suggests that mAbs more likely to be actively recycled by FcRn can also be cleared via the lymph, but at a slower rate than those less likely to be taken up into endothelial cells via pinocytosis or that fail to escape the endosomal/lysosomal clearance pathway via FcRn. Kraft et al. (2020) reported that a combination of assays that infer pinocytosis (i.e., heparin binding and/or PEG precipitation) and FcRn recycling enable identification of molecules likely to undergo rapid, non-target-mediated clearance in vivo. The relationships observed between the model output parameters and the experimental physicochemical attributes support the findings of Kraft et al. (2020) and contribute to a level of confidence in the ability of our subcutaneous dMVN and lymphatic models to predict subcutaneous absorption.

An important drug development application of an in vitro subcutaneous model would be the ability to inform on human subcutaneous bioavailability, a pharmacokinetic outcome that is highly variable and difficult to predict with available preclinical models. This is due in large part to the complexity of determining factors contributing to presystemic drug clearance (CL) and the overall rate of molecule absorption (Richter et al., 2012; Sánchez-Félix et al., 2020). The models developed here to facilitate these studies provide unique tools to measure specific drug interaction parameters likely impacting subcutaneous absorption and CL that would otherwise be challenging to obtain. However, our models still lack many components required to successfully predict bioavailability, including lymphatic transit and pre-systemic catabolism by resident immune cells (Richter et al., 2012). Future work determining avenues to integrate dMVN and lymphatic measures into physiologically based modelling approaches would be one route to overcome this constraint (Hu and D’Argenio, 2020). Such efforts should utilize compounds with strong clinical data due to poor cross-species translation of subcutaneous pharmacokinetics (Richter et al., 2012). This was an additional limitation of our preclinical mAb panel in that we only possessed pharmacokinetic parameters derived from rodent studies.

In conclusion, our current dermal and lymphatic microvessel models offer a focused window of observation into a hypothetical drug depot site and allow differentiation of protein behaviors potentially influencing bioavailability. Future efforts to combine both dermal and lymphatic components within a single MPS possessing net convective flow through a microfluidic pump (Offeddu et al., 2021b) and/or relevant resident immune cells would offer even greater insight into the processes governing SC depot absorption and cellular interactions influencing drug clearance. The use of cells from different individuals to make patient-specific MPS has been previously demonstrated (Offeddu et al., 2021c) and could, in the future, also be applied to the models considered here to assess subcutaneous drug distribution across different human populations.
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Conflict of interest
R.D.K. is a co-founder of AIM Biotech that markets microfluidic systems for 3D culture and receives research support from Amgen, Roche, Glaxo-Smith-Kline and Boehringer-Ingeheim.

Data availability
The datasets generated during the current study are available from the corresponding authors on reasonable request.

Author contributions
G.S.O., M.A.B., S.H., H.D., K.C., and R.D.K. designed the studies; G.S.O., J.S., Z.W., M.A.B., S.H., and S.W.C. performed the experiments. G.S.O., M.A.B., S.H., and K.C. wrote the first draft of the manuscript, and all authors contributed to its final form and scientific discussion.

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