Development and characterization of an *in vitro* method for investigating the pharmacokinetic behavior of long-acting subcutaneously injected therapeutics

Developing an artificial extracellular matrix suited for extended-release (> 1 week) within the subcutaneous injection site simulator n=3 (SCISSOR N3)

Striking differences have been observed in measured bioavailability between pre-clinical models and human clinical outcomes following subcutaneous (SC) delivery, particularly in the case of biopharmaceutical drugs. To this end, the subcutaneous injection site simulator (SCISSOR) was designed and initially validated to better mimic the human subcutaneous extracellular matrix environment with a series of monoclonal antibodies. We present data for a range of pharmaceutical motifs examined in the SCISSOR, and describe modifications made to the assay format to increase the time in which an API can be monitored from 1 day to 1 week, expanding the range of applications for this *in vitro* method.

INSTRUMENTAL ANALYSIS

SCISSOR Assay of Therapeutics

Multiple formulations containing caffeine, insulin, denosumab, and superpositive (+) and supernegative (-) green fluorescent protein (GFP) were tested using the SCISSOR system. Concentrations of injectate in the receiving chambers were monitored in real-time using *in situ* fiber optic dip probes connected to the Rainbow UV-Vis spectrometer (Pion Inc.), and offline analysis was carried out with a FLUOstar® Omega plate reader

Material Characterization of Artificial Extracellular Matrices

The rheological characteristics of the SCISSOR artificial extracellular matrix (ECM) and extended release artificial extracellular matrix (ECM-XR) were analyzed before and after assay using an Anton-Paar® MCR102e rheometer.

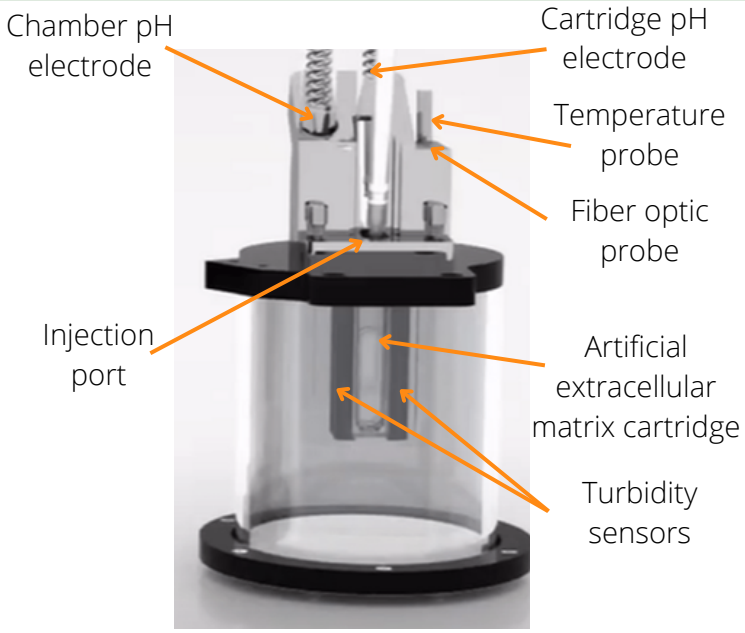

Figure 1. The prototypical SCISSOR N=3 assay setup with the addition of Pion's Rainbow® fiber optic probes®

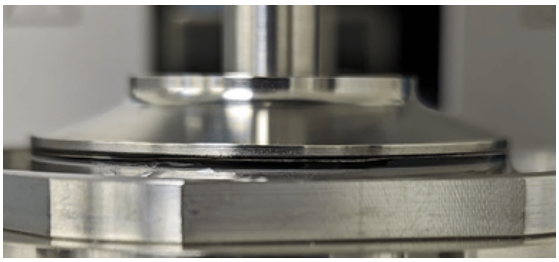

Figure 2. ECM-XR sample loaded onto an Anton-Paar MCR 102e rheometer with 25 mm plate-plate geometry

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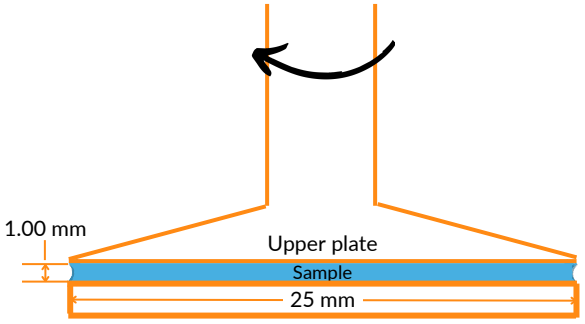
Material Characterization of Artificial Extracellular Matrices

Theoretical Background

To understand the mechanical differences between the ECM and ECM-XR, we sought to quantify the loss and storage modulus (and therefore, complex viscosity) of each type of artificial extracellular matrix. Our rheometer stage featured a 25 mm plate-plate geometry, which was used during an oscillating frequency sweep at 1% strain from 0.1-100 Hz. During this analysis, the rheometer oscillates the upper plate back-and-forth over a 3.6 degree range (1% of 360 degrees), at a variable rate of 0.1-100 times per second. The instrument can measure the force it takes to move the upper plate over that range in order to calculate how much the ECM is impeding that motion. (see **figure 3** and [1] for expanded detail).



1. $T = F \cdot r \sin(\theta)$
2. $\tau = \frac{F}{A}$
3. $\dot{\gamma} = v/h$
4. $\eta = \frac{\tau}{\dot{\gamma}}$

Figure 3. (top) General schematic of the measuring rheometer stage used in this study. (bottom) Equations 1-4 show how viscosity can be calculated in a general plate-plate rheometer experiment. Equation 1 shows how the force (F), radius (r), and magnitude of movement (θ) is used to solve for the torque (T) needed to produce fluid flow. Equation 2 shows how F and the surface area (A) in contact with the sample is used to calculate the shear stress (τ). Equation 3 The shear strain (γ) is solved for by using the velocity of fluid flow (v) and the gap height (h, 1.00 mm in the example above). Finally, equation 4 shows how equations 2 and 3 are used to calculate the viscosity of a sample. For expanded detail, see [1-2].

Data Analysis

The results of the analysis previously described can be seen in **table 1**.

The ECM had a 99% decrease in complex viscosity, while the ECM-XR was able to conserve ~20% over a long-term experiment. This was mainly due to the ECM-XR's ability to retain the ratio of storage-to-loss modulus, while the ECM lost all material characteristics responsible for its storage modulus. The large deviation in loss modulus of the ECM is due to the instrumental limitation of lower viscosity readings given the 25mm plate-plate measuring geometry (Figure 3). [1,2] Overall, the ECM-XR demonstrated its mechanical fitness for longer (1-2 week) experiments within SCISSOR.

Prototype	Complex Viscosity (Pa·s)	Storage Modulus (Pa)	Loss Modulus (Pa)
ECM	1.8 ± 0.3	0.45 ± 0.8	1.6 ± 0.3
ECM-XR	3.3 ± 0.3	2.9 ± 0.3	0.4 ± 0.1

>100 hours

Prototype	Complex Viscosity (Pa·s)	Storage Modulus (Pa)	Loss Modulus (Pa)
ECM	0.005 ± 0.002	0.002 ± 0.002	0.004 ± 0.002
ECM-XR	0.6 ± 0.1	0.5 ± 0.1	0.16 ± 0.08

Table 1. Rheometric characteristics of ECM and ECM-XR before and after assays lasting >100 hours. N=4 or 5 for the pre- and post-assay measurements, respectively.

[1] Mezger, T. G. (2018). Applied Rheology with Joe Flow on Rheology Road. Austria: Anton Paar GmbH.
[2] https://wiki.anton-paar.com/us-en/basics-of-rheology/

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Formulation Injections

Theoretical Background

Previously, the ECM was validated using a range of monoclonal antibodies to show how the *in vitro* rank-order release profiles from SCISSOR correlate with clinical data. [3] To build on this data set, a small molecule, peptide, protein, and monoclonal antibody were injected into both the ECM and ECM-XR. In most cases, to monitor the release of "API", data was collected *in situ* using Rainbow fiber optic probes. Absorbance spectra were collected from 200-700nm every 15 seconds for the first 2 hours of each experiment, then every 5 minutes thereafter. Each "API" required a specific analysis method to quantify the concentration of injectate in the receiving chamber over time. (**Table 2**) The release profiles for each are shown in the sections below.

Injection	Instrument	Analysis Method Details
Caffeine	Rainbow	Integrated the 2nd derivative of the absorbance profile between 262-284 nm
Rapid Insulin	Rainbow	Integrated the 2nd derivative of the absorbance profile between 280-320 nm
Basal Insulin	Rainbow	2nd derivative absorbance at 282 nm
+/- GFP	FLUOstar® Omega	Monitored fluorescence at λex = 480 nm and λem = 520 nm
Denosumab	Rainbow	Integrated the 2nd derivative of the absorbance profile between 266-292 nm

Table 2. Methods of analysis for each injection type

Release Profiles

Caffeine

Caffeine is a small polar molecule (molecular weight = 194.19 g/mol) that diffuses quickly in a variety of human tissue. [4] To validate similar behavior with both the ECM and ECM-XR, 50 µL of 5 mg/mL caffeine solubilized in **10 mM phosphate buffer (Pion Inc.)** was injected into each artificial extracellular matrix. The resulting % release-over-time data is shown in **figure 4** below. The overlapping spectra demonstrate that both the ECM and ECM-XR can evaluate molecules that naturally diffuse quickly in the human subcutaneous space. This is important, as formulation scientists would like labile molecules within formulations to all act similarly in a simulated SC environment. Chamber 2-ECM exhibited early release, which has a small chance of happening during injection into any SCISSOR artificial extracellular matrix, as reflux up the needle pathway is a common mechanism of complication during pharmaceutical injection. [5]

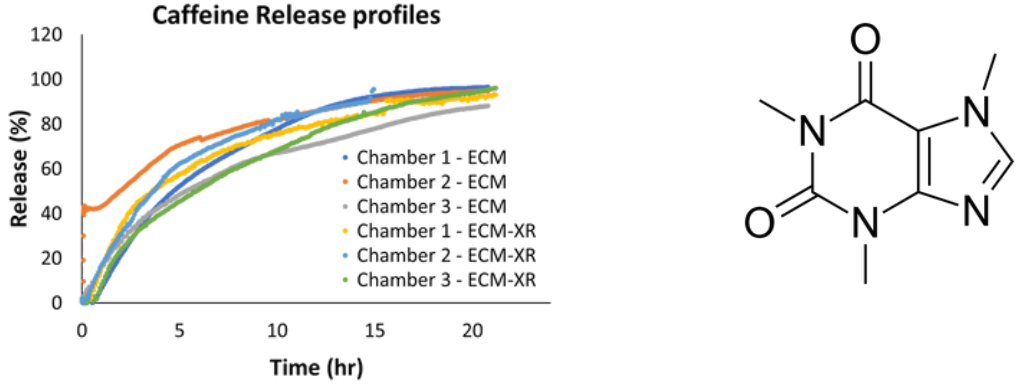

Figure 4. (left) % release profiles for 2 SCISSOR N=3 experiments, with ECM and ECM-XR. Each run is denoted by a color and labelled by which chamber and artificial extracellular matrix was used in each. (right) The molecular structure of caffeine.

[3] Bown, H. K., Bonn, C., Yohe, S., Yadav, D. B., Patapoff, T. W., Daugherty, A., & Mrsny, R. J. (2018). In vitro model for predicting bioavailability of subcutaneously injected monoclonal antibodies. J Control Release, 13-20.

[4] Levitt, D. (2013). Quantitation of small intestinal permeability during normal human drug absorption. BMC Pharmacol Toxicol, 14-34.

[5] Turgut, B., Demir, T., & Ulku, C. (2009). The Effects of Injection Site on the Reflux Following Intravitreal Injections. J Clin Med Res, 280-284.

RESULTS

Superpositive and Supernegative Green Fluorescent Protein

Superpositive (+) and supernegetive (-) GFP [6] are oppositely charged analogs of the same 27 kDA protein that appears green to the human eye. +/- GFP solubilized in phosphate buffer were utilized in this study to show how significantly polar molecules of a slightly larger size (~5x larger than caffeine) interact with both the ECM (200 µL injection) and ECM-XR (50 µL injection). Not only can we explore the chemical influence of each artificial extracellular matrix, but the larger size may show architectural influences not seen with caffeine.

Although the ECM and ECM-XR both have primarily negatively charged environments, and much like the human subcutaneous space, the ECM changes rapidly over time due to active flow. However biomimetic this behavior may be, long-term experiments will require a much more consistent environment over such a time frame. Because of this, a +GFP molecule should not diffuse out of the injection site of an artificial extracellular matrix tuned for extended-release profiles (>48 hours). As we can see in **figure 5**, the significant and immediate release from the ECM was due to flowability of the material, unlike the ECM-XR which tightly complexed the +GFP and didn't allow diffusion out of the cartridge.

The -GFP injections show significantly more release in both the ECM and ECM-XR, due to containing a like-charged molecule. In the case of the ECM, which is tuned to show immediate effects within 24 hours, the -GFP exhibited 50% release within the first day. The ECM-XR exhibited <10% release over a similar time frame. This is most likely due to the smaller theoretical pore size of the ECM-XR that allows for it's prolonged stability. Although the molecule is like-charged, the consistent and stable architecture of the ECM-XR results in much slower diffusion. A small amount of immediate release is likely caused by the labile components of the ECM-XR traveling through channels within the stiffer structure, similarly to that of a capillary system. When comparing the release of the + and - GFP molecules, each artificial extracellular matrix demonstrated the expected result, with -GFP exhibiting faster and greater overall release in both matrices as compared to the +GFP.

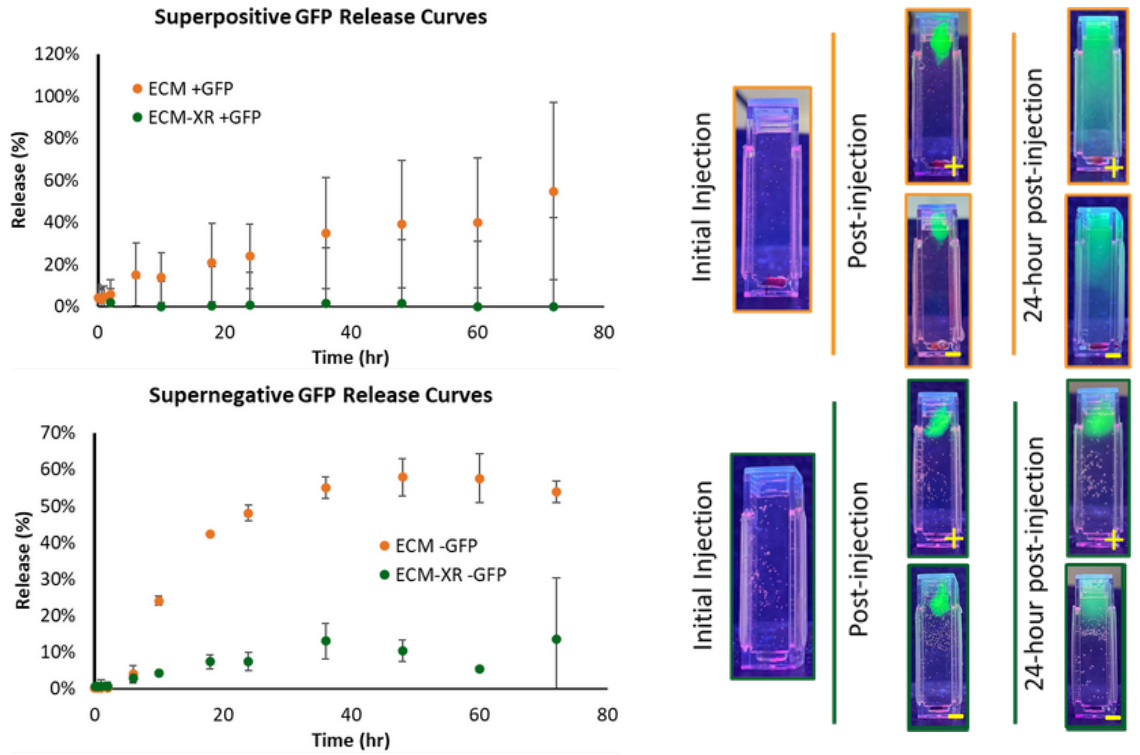

Figure 5. (left, top) Release profiles from the ECM (orange) and ECM-XR (green) of +GFP. N=3. (left, bottom) -GFP release profiles from the ECM and ECM-XR at given time points. N=3. (right) Representative images of -/+ GFP (labelled, yellow) diffusing through the ECM (orange) and ECM-XR (green) over 24 hours.

[6] GFP constructs were produced by the Biotech Suite at the University of Bath from plasmids kindly provided by Dr David Liu (Lawrence, M., Phillips, K., & Liu, D. (2007). Supercharging proteins can impart unusual resilience. J. Am. Chem. Soc., 10110-10112.)

Because the +/- GFP exhibit a colorimetric effect in the visible region, we were able to optically capture the injection behavior over a 24 hour period. The images representing what the ECM and ECM-XR look like before, immediately following, and 24 hours after injection can be seen above in figure 5. Before injection, both the ECM and ECM-XR are optically clear (>90% transmittance, data not shown). This is important because the SCISSOR N3 is outfitted with cameras and turbidity sensors which collect data in the visible region to supply researchers with *in situ* information about scattering and absorption events occurring around the injection site. We take advantage of this clarity to show how both the ECM and ECM-XR allow bolus formation during injection for oppositely charged molecules of interest.

The differences between each artificial extracellular matrix can be seen after 24 hours of assay, where we can observe increased diffusion of -GFP as compared to +GFP due to the oppositely charged injectates feeling contrary potentials within the cartridge. As with the release profile, the -GFP exhibits much more diffusion throughout the ECM and ECM-XR. As figure 5 depicts, the -GFP injection doesn't diffuse deep within the ECM-XR like it does in the ECM. This is most likely due to the smaller theoretical pore size in the ECM-XR as compared to the ECM, which has a highly mobile architecture. This is expected of the ECM-XR, as it's structure results in a higher storage modulus, as elaborated on above.

Rapid and Basal Insulin

Commercially available formulations of rapid insulin (50 µL injection, 3.5 mg/mL) and basal insulin (50 µL injection, 14.2 mg/mL) were assayed within both the ECM and ECM-XR to probe how each type of artificial extracellular matrix will express release profiles for peptide formulations designed to shorten or prolong API release, respectively. In **figure 6** The similarities between the ECM and ECM-XR are highlighted by the similar release profiles of the rapid release insulin, both exhibiting 100% release. Similarly to caffeine, molecules with minimal interaction with the surrounding environment will diffuse fastest, further validating the interactions with both the ECM and ECM-XR and molecules that should diffuse in ≤10 hours. The ECM-XR showed a significant difference between the rapid and basal insulin release patters.

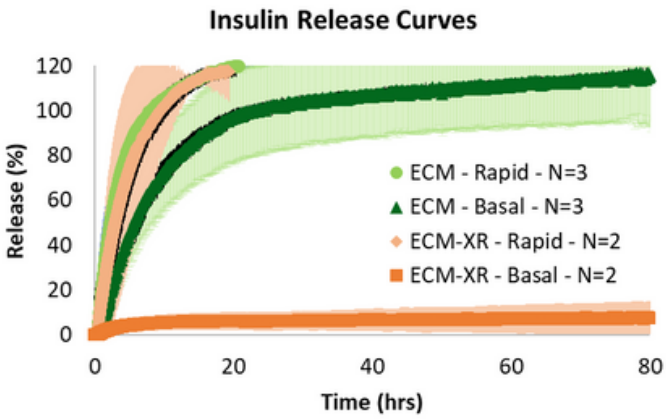

Figure 6. Release profiles of rapid and basal insulin in the ECM (light and dark green, respectively) along with the release profile of rapid and basal insulin in the ECM-XR (light and dark orange, respectively).

The basal insulin formulation is designed to release 3-4x slower than the rapid insulin formulation. This response was much more exaggerated in the ECM-XR than the ECM. The exaggerated difference shows that the ECM-XR's changes in chemical and mechanical architecture can go beyond what is appropriate when comparing formulations designed to be significantly different. As expanded on in previous publications, the SCISSOR system is designed to qualify rank-order release behavior of formulations, and the insulin release curves from the ECM correlate appropriately with previously published results. [1,7-8]

[7] Berger, M., Cuppers, H., & Hegner, H. (1982). Absorption Kinetics and Biologic Effects of Subcutaneously Injected Insulin Preparations. Diabetes Care, 77-91.
[8] FDA. (2007). LEVEMIR® (insulin detemir) [rDNA origin] Injection. USA: The Food and Drug Administration.

RESULTS

Denosumab

To reconfirm monoclonal antibody validation in the ECM and probe the behavior of monoclonal antibody release out of the ECM-XR, we injected 50 µL of a commercially available denosumab formulation into each artificial extracellular matrix, and monitored release over multiple days. The clinical data for this specific formulation reported that maximum release was achieved within at 10 days (3-21 day range). [8]

Release profiles from the ECM showed 100% release within 3 days (± 6 hours), with a consistent release rate up to the 2nd day. After that, there appears to be a 2nd pharmacokinetic step responsible for release until the end of assay which was not explored in the breadth of this study.

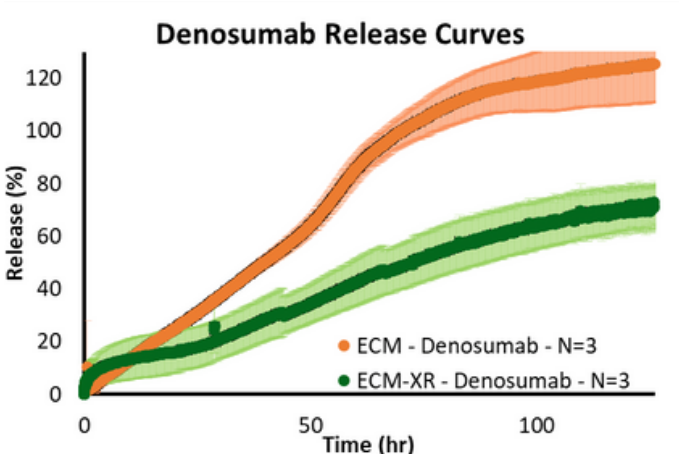

Figure 7. Relative release profiles of a commercially available denosumab formulation from the ECM (green) and ECM-XR (orange). N=3.

The release profile produced from injection into the ECM-XR showed a significantly prolonged profile, with a plateau being observed at ~160 hours. This prolonged injection is precisely the objective of the ECM-XR - conserving the chemical reactivity of the ECM while translating the experimental window from days to weeks. With the range of clinical data showing a maximum release between 3-21 days, both cartridges exhibit appropriate release profiles, and the correct choice would be dictated by how developers are wanting to translate the release kinetics.

Conclusions

Two artificial ECMs (ECM & ECM-XR) were evaluated using 5 model injectables within Pion's subcutaneous injection site simulator (SCISSOR). The release profiles were compared to show how each model released over smaller time scales, while the ECM-XR could sustain release over 1 week.

Caffeine injections into each model showed similar behavior over short time scales for both the ECM and ECM-XR, demonstrating the ECM-XR's analogous behavior in shorter release studies.

Injections of +/- GFP showed that the ECM-XR could appropriately complex +GFP for days, as opposed to the ECM release over the same time frame, demonstrating the expanded experimental timeframe of the ECM-XR.

Lastly, commercially available formulations of insulin analogs and denosumab were injected to elucidate peptide and monoclonal antibody release behavior within the ECM-XR.

In conclusion, although both the ECM and ECM-XR correctly model the environment of the human subcutaneous space, the ECM-XR demonstrated prolonged release profiles (up to 1 week) for the injections presented within this study – including small molecules, peptides, proteins, and monoclonal antibodies.

[8] FDA. (2007). Prolia® (denosumab) injection, for subcutaneous use. USA: The Food and Drug Administration.

This content was originally presented as a poster at the CRS conference and was authored by Conor Gomes, Mark Bradshaw, Imogen Anastasiou, and Balint Sinko of Pion Inc., and Kate Gridley and Randall Mrsny of University of Bath.

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