APPLICATION NOTE

Demonstrating the Excipient Effect *In Vitro* for Subcutaneous Pre-Clinical Formulation Analysis

The Pion SCISSOR N3 (right) and Rainbow R6 (left) set up to monitor *in situ* release of a formulation.

Designing subcutaneous (SC) formulations presents a complex challenge within the pharmaceutical development landscape, where achieving optimal drug stability, solubility, and bioavailability must be balanced with patient comfort and dosing constraints.

Traditionally, formulation composition - such as buffer systems, excipients, and pH - has been selected based on historical precedent and limited preclinical studies. However, subtle changes in formulation components can significantly influence drug performance, even before administration.[1,2]

As the demand grows for stable high-concentration SC therapies, especially for biologics and long-acting modalities, there is increasing recognition that rational formulation design must be informed by a deeper mechanistic understanding of drug release.[1] The importance of mimicking the human SC space has been shown, and is likely the reason that more complex biological drug formats deviate from animal studies when compared to the clinical results.[3-6] In this study, we employ the Subcutaneous Injection Site Simulator (SCISSOR N3™), an *in vitro* platform designed to more closely mimic the human SC environment, to evaluate and compare the effects of varying excipients can have on drug release when tested *in vitro*.[3-6]

METHODS

To examine how different formulation components affect monoclonal antibody (mAb) performance within the SCISSOR N3 platform, we used a commercially available formulation of denosumab (60 mg/mL), and independent excipients of polysorbate 20 (0.1 mg/ml, surfactant), and sorbitol (47 mg/ml, cryoprotectant) in phosphate (pH=7.4) or acetate buffer (pH 5.2).

The commercial formulation was dialyzed with 10kDa cutoff dialysis cassette (Slide-A-Lyzer G3 Dialysis Cassettes, 10K MWCO, Thermo A52971) against an acetate buffer (pH 5.2) or PBS buffer (pH 7.4), with combinations of the excipients included/excluded (polysorbate 20, sorbitol; Table 1).

In addition, a blinded mAb - mAbX (147 mg/mL, pH 5.4) –

Table 1. Details outlining the resulting environment for each dialysis experiment to prepare the denosumab formulations.

Formulation	Denosumab (not dialyzed)	Denosumab (dialyzed)	Polysorbate 20	Sorbitol	Acetate buffer	PBS Buffer
Pristine	60 mg/mL		0.1 mg/mL	47 mg/mL	pH 5.2	
Denosumab – All Excipients + Acetate – 18 hours		18 hours	0.1 mg/mL	47 mg/mL	pH 5.2	
Denosumab – All Excipients + Acetate – 3 days		3 days	0.1 mg/mL	47 mg/mL	pH 5.2	
Denosumab – All Excipients + PBS – 3 days		3 days	0.1 mg/mL	47 mg/mL		pH 7.4
Denosumab – No Excipients		18 hours			pH 5.2	
Denosumab – PS20		18 hours	0.1 mg/mL		pH 5.2	

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was tested in a similar format. The mAbX formulation was not dialyzed, and instead, different excipients were added to investigate the impact this addition had on performance (polysorbate 20, polysorbate 80, benzyl benzoate glycerol and sorbitol).

In vitro SC injection was analyzed using the SCISSOR N3 platform (Pion Inc.), where samples were injected into a cartridge filled with an artificial extracellular matrix (ECM) suspended in a chamber filled with biorelevant bicarbonate buffer. Drug diffusion from the cartridge to the chamber via dialysis membranes simulated drug uptake into the blood and lymphatics. Quantification of drug diffusion to the outer chamber was conducted *in situ* via UV-Vis monitoring using a Rainbow™ platform (Pion Inc.) which measured concentration in real-time. The spectrophotometric data was further quantified but the AuPro software (Pion Inc.) to convert concentration into %Release. Cameras and LED turbidity sensors in the SCISSOR were used to monitor post injection behavior within the SC environment, however, the data is not shown in this work.

RESULTS AND DISCUSSION

Case Study 1 – Denosumab Formulation Modulation

To test the effect of the different excipient components on the release kinetics of the denosumab molecule *in vitro*, we dialyzed the commercial denosumab formulation against the formulation components outlined in table 1. Once all samples were prepared, we ran control experiments where the formulation was dialyzed against its commercial excipients. In doing so, we found that dialyzing for extended time frames (3 days) in either buffer (Acetate and PBS) had an impact on the release of the mAb when then injected into SCISSOR (figure 2). When dialyzed for only 18 hours, the release profile of the mAb exhibited similar kinetics (99 ± 6% at 52 hours) to the pristine denosumab formulation (100% at 53 hours), therefore, this dialysis incubation interval was used for the remainder of the study.[2]

Effect of Buffer and pH

To investigate the pH and buffering effect on denosumab release and stability, we exchanged the acetate (5.2 pH) buffer for a phosphate (7.4 pH) buffer (PBS), observing similar pharmacokinetic behavior between the acetate and phosphate buffers initially (figure 2). However, later in the assay, between 15-40 hours, we saw a deviation between the two, where the PBS sample dipped in spectroscopic response within the area of analysis. When these experiments were left for much longer time periods (2-5 days, data not shown), the spectroscopic data for the PBS experiment showed heavy indications of light scattering in the outer chamber, indicative

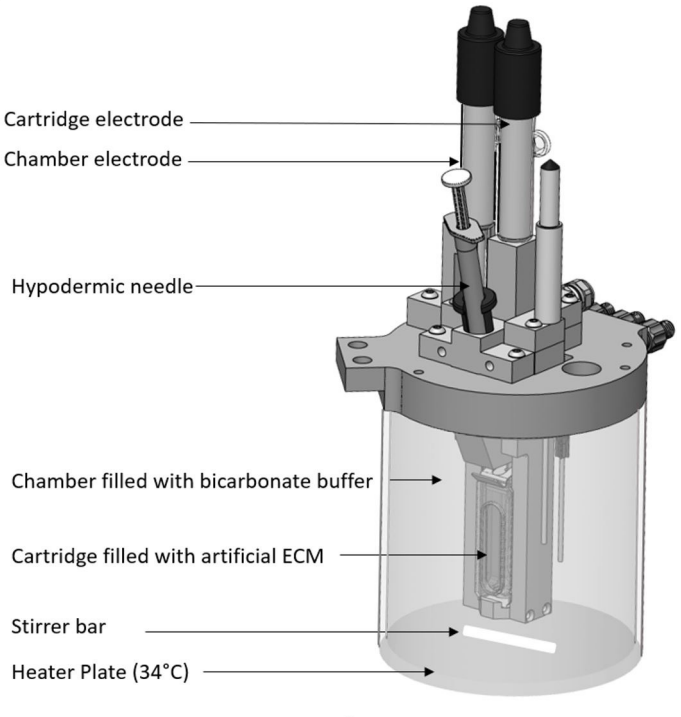

Figure 1. The Pion SCISSOR N3 chamber assembly labeled with tools and functionalities.

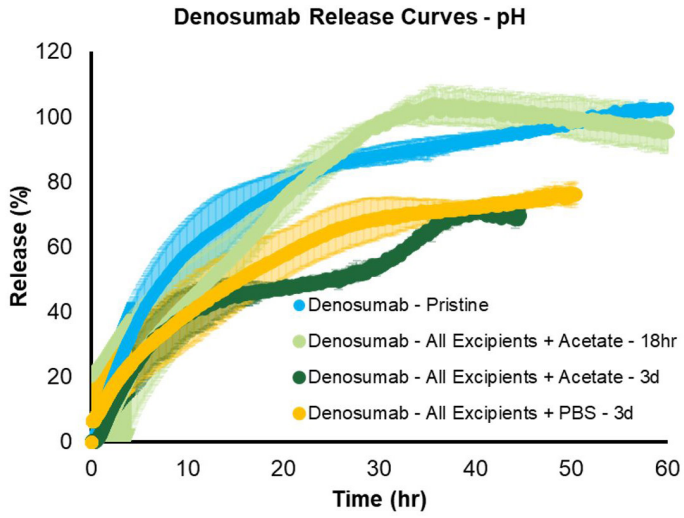

Figure 2. Denosumab release profiles (%) from the SCISSOR cartridge to the outer chamber as measured by Rainbow fiber optic UV. 200uL injections of a pristine denosumab commercial formulation (blue, n=2), denosumab formulation dialyzed against all excipients (acetate buffer) for 18hrs (light green, n=3), denosumab formulation dialyzed against all excipients (acetate buffer) for 3 days (dark green, n=2), denosumab formulation dialyzed against all excipients (PBS buffer) for 3 days (yellow, n=3). Error bars = standard deviation.

of some form of aggregation or solubility-changing event that would impact formulation performance *in vivo*.[2]

Excipient Effect

All additional excipients within the formulation (including polysorbate 20 and sorbitol) were systematically removed to demonstrate the impact the excipients have on the release profile and to act as a negative control. As expected, when all excipients were removed from the denosumab formulation, the formulation became more unstable and only 77% release was achieved by 52 hours (n=1) (figure 3, orange curve). We predict the reason for this slow-release profile is because the mAb begins to aggregate or precipitate at a faster rate than the commercial formulation, remaining at the injection site within the cartridge due to the lack of stabilizing/solubilizing excipients.

When only the polysorbate 20 (PS20) was present in the formulation, immediate release was seen for all injections, and a max release of 70 ± 12% was observed in less than 20 hours post-injection (figure 3, dark green curve). This early release is indicative of reflux up the needle pathway during injection into the SCISSOR cartridge. As polysorbate 20 is a surfactant, it decreases the viscosity and surface tension of the solution it is solubilized within, likely contributing to the formulation's increased tendency to flow up the needle pathway.

When sorbitol was added back into the formulation along with the PS20 (figure 3, light green curve), the %release recovered near to that of the original formulation. Considering this data, sorbitol was found to eliminate the early release characteristics from injection seen when only having PS20 present in the formulation by increasing the viscosity of the solution. To reiterate, the PS20 reduces the viscosity and surface tension of the injectate while sorbitol's characteristics as a thickening agent counteracts the ramifications of including PS20. In this experiment, we see that sorbitol not only acts independently as a cryoprotecting agent but also counteracts some of the issues associated with using surfactants to solubilize monoclonal antibodies for injection into an aqueous environment. Overall, this case study displays the drastic impact that each of the formulation components can have on the release kinetics of mAbs in SCISSOR and demonstrates the fitness of the SCISSOR and Rainbow platforms to track and probe the excipient effect during formulation development.[2]

Case Study 2 – mAbX: Blinded mAb Excipient Study

In our second case study, we investigated the effect of adding different excipients to an already existing mAb formulation. We used a blinded mAb, mAb X, in an undisclosed formulation at pH 5.8 (147 mg/mL). To this formulation, we added the following excipients: polysorbate 20 and 80 (which exhibit different lipo- and hydrophilicities), sorbitol, benzyl benzoate, and glycerol aiming to probe the role and impact that each excipient will have on mAb release in SCISSOR.[2]

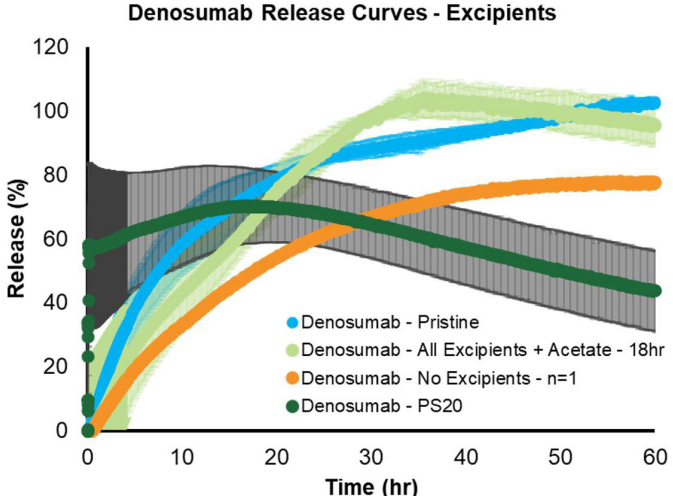

Figure 3. Denosumab release profiles (%) from the SCISSOR cartridge to the outer chamber as measured by Rainbow fiber optic UV. 200uL injections of a pristine denosumab commercial formulation (blue, n=2), a denosumab formulation dialyzed with both excipients present (sorbitol + PS20) (light green, n=3), the denosumab API without excipients (orange, n=1), and denosumab with only PS20 (dark green, n=2). Error bars = standard deviation.

Sorbitol

Adding sorbitol had marginal effects on the mAbX release profile compared to the pristine formulation with 59 ± 9% released at 50 hrs (figure 4). However, multiple inflection points were seen during release, resulting in a slightly higher

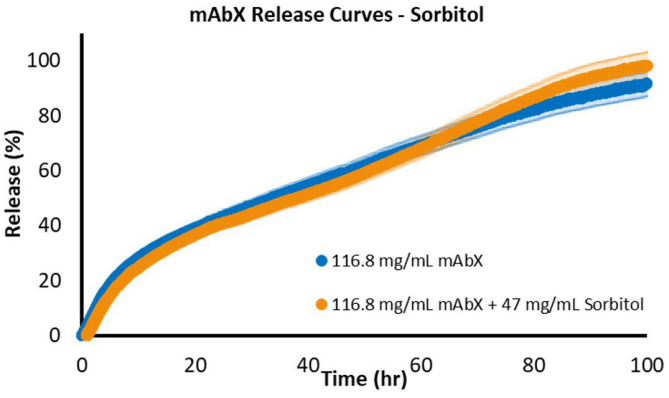

Figure 4. mAbX release profiles (%) from the SCISSOR cartridge to the outer chamber with and without Sorbitol as measured by Rainbow fiber optic UV. 200uL injections of a pristine mAb X (blue, n=3) and mAbX + 47 mg/mL Sorbitol (orange, n=3). Error bars = standard deviation.

response at 100 hours of 81 ± 5%.[5] This is expected, as sorbitol is a passive excipient, not acting on the API itself to aid physiochemically.

Polysorbate 20 & 80

Polysorbate is often added to formulations to act as a solubilization/stabilizing agent. For mAbX, we added PS20 and PS80 to observe the impact the different chain lengths have on release (figure 5). Similarly, as with denosumab, having PS20 as the only added excipient within the mAbX formulation caused immediate release in one of the triplicate injections completed. This is (again) likely attributed to the surfactant interrupting the surface tension that typically inhibits reflux up the needle pathway. However, when polysorbate 80 (PS80) was added instead, no immediate release was seen and the release curve mimicked that of the pristine mAbX control with 62 ± 2% release achieved by 50 hours. In this case, the PS80 did not significantly alter the release kinetics of the monoclonal antibody. PS80 appeared to reduce the likelihood of early release as compared to PS20, which had a 33 % chance of premature mAb release. This would likely qualify as initial evidence on which solubilizer to include in a prototype formulation. The reason for the difference in release between PS20 and PS80 addition is likely due to PS80's higher lipophilicity compared to PS20, as the attractive interactions with the stainless-steel needle may be weaker. Based on these *in vitro* observations, we could consider polysorbate 80 a more suitable solubilizer/stabilizer for maintaining controlled release profiles of this type of mAb.[2]

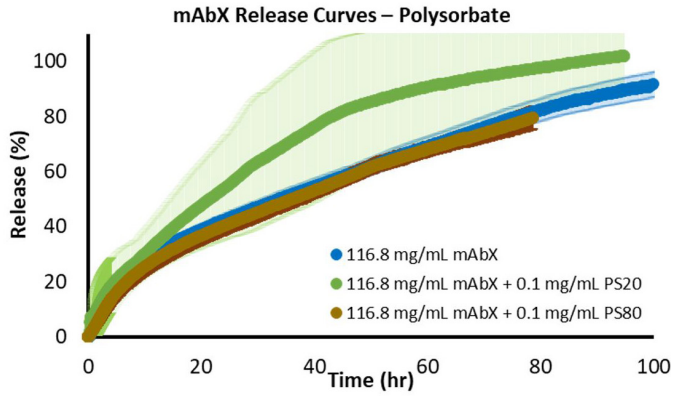

Figure 5. mAbX release profiles (%) from the SCISSOR cartridge to the outer chamber with and without Polysorbate 20/80 as measured by Rainbow fiber optic UV. 200uL injections of a pristine mAb X (blue, n=3), mAbX + 0.1 mg/mL Polysorbate 20 (green, n=3) and mAbX + 0.1 mg/mL Polysorbate 80 (brown, n=3). Error bars = standard deviation.

Benzyl Benzoate

Benzyl Benzoate is often added to formulations also to aid with API solubility/stability. In this study, we introduced benzyl benzoate at different pH's to observe the impact of the excipient and pH on the mAb performance. When benzyl benzoate was added to the mAbX formulation at pH 7.4, it resulted in a release curve which had multiple inflection points before 100% release that increased the standard deviation of each assay but still achieved 100% release at 70 ± 1 hours post-injection, significantly faster than pristine mAbX. When the pH of the same formulation was adjusted to pH 9, it slowed the release curve slightly as compared to the pristine mAb but did not result in any spectroscopic aberrations that would cause the higher standard deviation seen at pH=7.4.

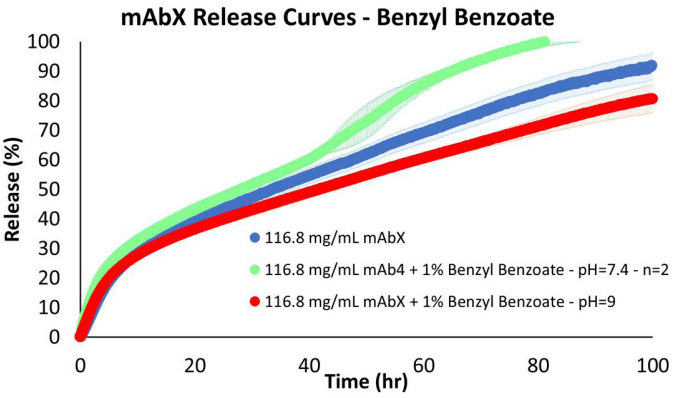

Figure 6. mAbX release profiles (%) from the SCISSOR cartridge to the outer chamber with and without Benzyl Benzoate as measured by Rainbow fiber optic UV. 200uL injections of a pristine mAb X (blue, n=3), mAbX + 1% Benzyl Benzoate (pH 7.4) (Green, n=2). and mAbX + 1% Benzyl Benzoate (pH 9) (Red, n=3). Error bars = standard deviation.

Glycerol

Another excipient commonly included in formulations is glycerol, often used for its stabilizing properties, as a thickener, or as a cryoprotectant. In this study, we added glycerol to the mAbX formulation at pH 7.4 which had a significant impact on the initial release of the mAb. The addition of glycerol appeared to significantly increase the release rate immediately following injection. However, this increased rate quickly levels off around 10 hours and is then surpassed by the pristine mAbX formulation. This is an unexpected behavior; glycerol is expected to increase the viscosity of the solution, and therefore, slow the rate of diffusion. It is a possibility that glycerol decrease the surface tension of the injectate when at a higher concentration (causing faster diffusion), then as the bolus is diluted, the diffusion of the formulation slows because there is now less API moving through a more viscous medium.

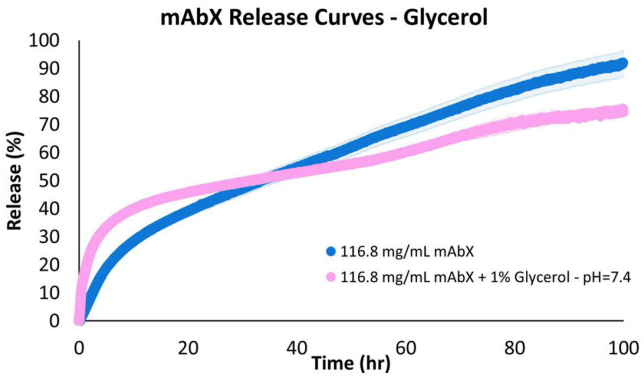

Figure 7. mAbX release profiles (%) from the SCISSOR cartridge to the outer chamber with and without Glycerol as measured by Rainbow fiber optic UV. 200uL injections of a pristine mAb X (blue, n=3) and mAbX + 1% Glycerol (pH 7.4) (pink, n=3). Error bars = standard deviation.

CONCLUSION

The SCISSOR platform demonstrates clear capability in distinguishing the effects that different excipients have on monoclonal antibody (mAb) release kinetics during and after delivery,[4-6] illustrating the ability to decide on suitability between analogous constituents.

As shown with mAbX assays, some excipients may need to be coupled with one another to gain the desired outcome. For example, including surfactants to aid in solubility would typically lead to an increased rate of API bioavailability. However, the introduction of excipients that reduce viscosity can lead to reductions in successful delivery of the bulk formulation, counteracting the benefits of increased solubility. Adding cryoprotectants not only aids in manufacturing and shipping barriers, these excipients (that typically increase viscosity) can counteract the negative effects of the solubilizers. It would require a set of assays to determine the correct excipient, as well as the correct concentration of the chosen molecule.

Our comparison of polysorbate 20 and polysorbate 80 highlights how similar excipients can lead to different outcomes. Polysorbate 80, likely due to its higher lipophilicity, showed more controlled release and avoided a tendency to travel up the stainless-steel needle pathway as compared to polysorbate 20. While these early results suggest polysorbate 80 may be the better option for this formulation, a more extensive study is needed to fully understand the performance differences between these two surfactants in subcutaneous formulations.

Whilst the excipients themselves are essential components of the formulation, we also demonstrate how SCISSOR can highlight the impact formulation buffer and pH play in release behavior. The Rainbow and AuPro platforms help provide a deeper mechanistic insight into the stability of API over time, however, this data is not shown within this report.

Gaining deeper insight into how excipients interact with specific biomolecules is critical for designing SC therapies that are both effective and consistent. Platforms like SCISSOR provide a valuable way to evaluate these effects *in vitro*, giving researchers a biorelevant, practical, and animal-free approach to formulation development.

Understand *in vitro* how a drug behaves after subcutaneous injection and the impact this has on drug release with Pion's SCISSOR N3.

About the SCISSOR technology

The SCISSOR technology, originally developed and validated primarily using mAbs, and now backed by 10+ years of experience in *in vitro* subcutaneous testing, is designed to mimic the human subcutaneous environment—enabling researchers to compare drug release kinetics and potential immunogenicity risk in a controlled setting. By providing insights into how a drug behaves post-injection, SCISSOR aids in optimizing formulations and predicting clinical outcomes without relying on animal testing.

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