The enclosed manuscript:

- Establishes the critical lack of knowledge regarding the *fate* of pharmaceuticals after intramuscular IM injection (as opposed to the abundance of articles discussing pharmacokinetics)

- Collates information and discusses merits/caveats of technologies described to model drug fate post-injection, to then apply to developing a novel IM-specific *in vitro* tool

- Proposes key components/properties of the IM injection site for use in a novel *in vitro* system, based upon evidence of IM distribution of injected material *in vivo*

- Summarises current FDA-approved IM pharmaceuticals (with known PK data) and their formulation type

Evaluating parameters affecting drug fate at the intramuscular injection site

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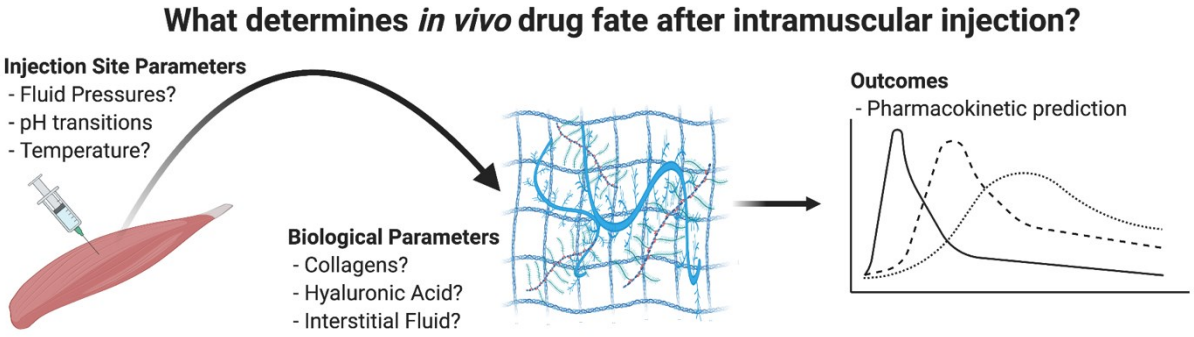
Abstract

Intramuscular (IM) injections are a well-established method of delivering a variety of therapeutics formulated for parenteral administration. While the wide range of commercial IM pharmaceuticals provide a wealth of pharmacokinetic (PK) information following injection, there remains an inadequate understanding of drug fate at the IM injection site that could dictate these PK outcomes. An improved understanding of injection site events could improve approaches taken by formulation scientists to identify therapeutically effective and consistent drug PK outcomes. Interplay between the typically non-physiological aspects of drug formulations and the homeostatic IM environment may provide insights into the fate of drugs at the IM injection site, leading to predictions of how a drug will behave post-injection *in vivo*. Immune responses occur by design after e.g. vaccine administration, however immune responses post-injection are not in the scope of this article. Taking cues from existing *in vitro* modelling technologies, the purpose of this article is to propose "critical parameters" of the IM environment that could be examined in hypothesis-driven studies. Outcomes of such studies might ultimately be useful in predicting and improving *in vivo* PK performance of IM injected drugs.



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Introduction

Oral drug delivery is considered the preferred administration route for a variety of reasons, but not all drugs are amenable to this approach [1]. Indeed, many drugs do not have the physicochemical properties required for oral delivery or are extensively modified by first-pass metabolism in the gut and/or liver in a manner that limits their efficacy. Such drugs are commonly administered by injection. While more cumbersome and/or unpleasant to the patient relative to oral dosing, injections have the advantage of accurate, direct, and rapid delivery [2]. Most injections are administered via one of three routes: into the vasculature (intravenous; IV), into the fatty space below the dermis (subcutaneous; SC), or into skeletal (striated) muscle tissue (intramuscular; IM). The IM injection site is well vascularized compartment that has been extensively used for the introduction of a wide variety of medicines including sedatives, anti-emetics, hormone therapies, analgesics, and immunizations [3]. There are multiple acceptable muscle bellies for IM injections, with administered formulations providing either rapid uptake or extended release pharmacokinetic (PK) outcomes [4].

It has been speculated that the medical procedure of IM injection existed as early as around 500 AD [5]. This method became common practice after the genesis of the antibiotic-age with the introduction of penicillin in the 1940s [6]. Originally a physician-specific procedure, IM injections became predominantly a nurse-specific role in the late 1960s that was associated with a paradigm shift in the role of nurses in delivering healthcare, and most recently to self-administration [6]. The IM route of administration can be associated with pain, either at the time of injection and/or after some period due to cellular responses at the injection site, with a large fraction of IM injection literature focused on this issue. Inoculations induce immune responses by design for efficacy, but this article focusses on drug fate of therapeutics as opposed to vaccines, and will not cover immunological responses in depth. In the context of post-injection cellular responses, exposing patients to music and applying pressure to the site of administration have been used to “reduce pain” associated with IM injections [7, 8]. In the case of immunomodulation agents, such as interferon beta, “warm compresses” have been described as beneficial to patients [9]. Whilst this subjective knowledge could be useful for patient care during and immediately after administrations, the foremost question about IM injections has not been addressed. Despite the widespread use of the IM injection procedure and literature relating to IM PK, there is still limited knowledge regarding the fate of IM

injected materials following their administration and how such events might affect the performance of such medicines. There is a paucity of literature related to IM injection site events that could affect the fate/actions of an injected drug.

While IV delivers a drug directly into the systemic circulation, SC and IM injections deposit a drug formulation at a site in the body where local events could limit and/or modify the movement of the injected drug into the systemic circulation. Events associated with this process in humans are not predicable from pre-clinical *in vivo* SC injection models, and extensive efforts have been made to provide reliable *in vitro* models to predict drug fate following such an injection [10]. Currently, there is no pre-clinical *in vivo* model that is acknowledged as predicting IM injection site outcomes in man. The focus of this article is to examine likely properties of the human IM injection site, in order to identify parameters that might affect drug fate and be useful in providing reliable *in vitro* models to predict IM injection site events and outcomes.

Overview of the IM injection site

The fundamental function of skeletal muscle is to physically support the skeleton of an organism in a manner that allows movement, i.e. “functional independence” [11]. Approximately 40% of the total human body mass is composed of skeletal muscle [11, 12]. To enable repetitive and sustained movement, skeletal muscle is heavily vascularised to provide sufficient glucose and oxygen for the metabolic demands of both glycolysis and mitochondrial respiration [13, 14]. A by-product of the metabolism constantly occurring in skeletal muscle associated with posture and breathing, skeletal muscle is a critical contributor of maintaining homeostatic core body temperature [11]. IM injections deposit a drug formulation directly into the belly of a large-mass skeletal muscle. The most common sites are the dorsogluteal, ventrogluteal, vastus lateralis, rectus femoris, and deltoid muscles [15, 16]. A key advantage of IM versus SC deposition of drugs is the superior maximum injection volume “not exceeding” 5 mL for single IM injections (depending on the choice of muscle belly), compared to 1.5 mL maximum for SC injections [17-21]. However, large volume injections exceeding 3 mL are not frequently used, being reserved for pharmaceuticals such as fulvestrant [15, 22]. Indeed, the British National Formulary has suggested that no more than 3 mL of material be injected at any one IM site [23]. Additionally, the IM environment is considered to be more vascularized than the SC environment. This difference is exemplified by the observation that

peak serum follicle stimulating hormone levels were higher and occurred earlier after IM injection than after SC injection [24]. A similar finding of more rapid systemic uptake following IM versus SC has also been observed for insulin [25].

IM injections are achieved via a needle that penetrates the skin and adjacent fatty subcutaneous layer before terminating within the underlying skeletal muscle [16, 19]. Skeletal muscle has a highly organised structure, with the lowest level of the functional organisation within skeletal muscle being fibres collectively formed from myofibrils (**Figure 1**). These muscle fibres are bundled into fascicles [11]. In turn, fascicles form the whole skeletal muscle organ, with each level of this organisation being supported by layers of acellular, protein-based material known as the extracellular matrix (ECM). Muscle fibres are enveloped by the endomysium, fascicles by the perimysium, whilst the entire muscle is covered by the epimysium; all of these coverings are composed of discrete ECM components organized specifically for each functional element [26]. Endomysium layers are typically cylindrical, as they form around individual fibrils [27]. Perimysium layers are thicker, comprising several individual collagen layers [27]. The dense, collagen rich epimysium also extends beyond the individual muscle organ to form attachments to skeletal elements via tendons. Together, these ECM layers can be collectively termed the fascicular fascia [28].

Studies examining the distribution of IM injected drugs have demonstrated that there is limited delivery into myocytes themselves, with drug distribution being observed extensively through the dense, highly vascularized ECM network of the fascicular fascia. Comparison of several small molecule drug classes administered into the rat rectus femoris muscle in either an aqueous or an oily formulation consistently showed spreading behaviour along fascicular fascial planes [29]. Distribution of fluorescently labelled vascular endothelial growth factor (VEGF) following IM injection into rat rectus femoris muscle showed it to exclusively localize to this ECM network [30]. Individual skeletal muscle fibres are typically provided with a dedicated blood supply via several capillaries [31]. Studies with VEGF highlighted the rich blood supply present within the fascicular fascia due to its binding to cognate receptors on vessels within the fascicular fascia (**Figure 2**) [30]. Thus, current data suggests that the fascicular fascia is the origin of IM injection site elements that might interact with pharmaceuticals in a manner that could affect systemic uptake.

Factors that could alter the IM injection site environment

As mentioned, IM-injected drugs are perceived to primarily experience a well-vascularized ECM network of the fascicular fascia prior to their uptake into systemic circulation. Before considering the nature of this environment with regard to events following an IM injection, it is worth considering if the act of injection itself might significantly alter the nature of this environment. For example, significant needle-induced physical tissue trauma could result in an environment that deviates from an environment dominated by ECM components. One way to examine this possibility is calculate the extent of tissue coring from a typical IM injection. One can approximate the volume of striated muscle affected by assuming a 24-gauge needle (0.4 mm radius and $\simeq$ 38.1 mm length) being fully inserted into the muscle. We can use the full needle length as a worst-case scenario, since the layer of SC fat affecting depth of penetration varies widely from person to person, thus making it unlikely that the full length of the needle was inserted into skeletal muscle (**Equation 1**).

Equation 1. $$38.1\ \mathrm{mm} * (\pi * 0.4\ \mathrm{mm}^2) \simeq 19.2\ \mathrm{mm}^3$$

In this scenario, the calculated total volume of damaged muscle tissue due to needle coring would be roughly 19.2 mm^3. Considering that an IM injection volume could be up to 5 mL, this amount of damage does not appear to be of consequence in significantly altering the IM injection site properties. This consideration assumes, however, that no significant blood vessel damage was induced by the needle track. Such an event could result in a larger volume of material at the injection site that is unrelated to the drug formulation. Additionally, there is the potential for subsequent serum-associated exudate that might enter the injection site over the next few hours to days as a result of inflammatory events initiated by the drug formulation (something which is problematic to assess). While therapeutic agents would be formulated to minimize such inflammatory events, most vaccines are designed to stimulate these processes in order to be efficacious.

The well-vascularized nature of the IM injection site has been used effectively for drug delivery where rapid uptake into the systemic circulation is desired, but the large volume available for IM injections provides opportunities for long-acting injectables (LAIs) for various indications [32-35]. As such, potential changes to the IM environment induced by the injection event might have less of an effect for a rapid uptake compared to events associated

198 with LAIs that are typically designed to form a depot and release drug over an extended period
199 of time (usually weeks to months) in a controlled manner [36-39]. Examples of LAI format
200 therapeutics include antipsychotics and antiretrovirals, such as olanzapine and cabotegravir,
201 respectively [38, 40, 41]. **Table 1** highlights the range of both short-acting and LAI drugs
202 currently FDA-approved for IM delivery. The total lifespan of the depot itself has been
203 suggested to be dependent on the molecular weight and concentration of the administered
204 drug [42]. One rationale behind LAI use is to ensure consistent exposure of medicines where
205 omission to take a daily oral tablet, for example, could have a deleterious therapeutic impact
206 [43, 44]. If the IM injection site environment changes over the time course of drug release
207 from a LAI depot, however, this might result in a deviation from the anticipated exposure
208 profile. Thus, a fundamental question to be addressed when trying to model the IM injection
209 site is: do elements of the ECM environment affect interactions between drug release and
210 uptake into the systemic circulation? To date, the literature has focussed on drug PK profiles
211 following IM injection, but not on events at the injection site that might affect these outcomes
212 [45-60].
213
214 **Transition events at the IM injection site**
215 When we speak of fate, we refer to events experienced by the formulation that affect the
216 rate and duration of drug release from the IM injection site and the ability to reach the
217 intended drug target(s) in the body. IM drugs are commonly delivered in non-physiological
218 formulations and there are potential events that might occur as the body undertakes the
219 process of returning the injection site to its original homeostatic state. Events associated with
220 this transition from "formulation state" to "homeostatic state" could be affected by skeletal
221 muscle physiology, ECM components, and properties of the injected formulation (such as pH,
222 viscosity and solubility) [61-64]. Interplay between non-physiological formulation
223 properties/components and the homeostatic IM environment could therefore dictate drug
224 fate post-injection *in vivo*. *In vitro* models to characterize the fate of drug formulations
225 intended for IM injection will require acknowledgement of the potential impact of these
226 transition events if they are to ultimately be used to understand and possibly predict *in vivo*
227 outcomes in patients. Here, these entities within skeletal muscle are artificially catalogued
228 alongside their potential impact on drug fate in **Table 2**.
229

Introducing immunologically-"foreign" material will induce a reaction by local cells in the host. Inflammation as a result of this response would result in post-injection "pain" for patients. However, certain cases demand such a response; inoculations induce immune responses by design to ensure their efficacy. Further immunological responses include the formation of fibrous capsules around implants in tissues which could, in theory, extend to depots of LAI pharmaceuticals [65]. In the context of developing a novel *in vitro* IM-predictive tool, however, modelling such processes would be technically challenging and prohibitively costly. In the context of this review, we therefore acknowledge that such events can occur, but the non-immunological transition events and conditions will be focussed upon hereon.

When injecting pharmaceuticals into the human body, the non-physiological characteristics of the injected material are at odds with the physiological conditions of the local tissue. The host conditions are maintained by homeostatic processes which are essential for survival. The injectable material, on the other hand, is not necessarily analogous to these conditions. The material can be non-aqueous (e.g. oil-based) or acidic/alkaline, for example [63, 66-68]. Once injectable material has been deposited into the host environment, the fundamental conditions of the immediate environment (such as pH) may be altered due to the properties of the injectable. Over time, the host environment then resolves to the homeostatic state. As a result, the injected material will gradually transition from the formulation-state to the homeostatic-state of the host. Furthermore, the deposited material must adapt to the local space as it disperses. The literature indicates that the spread/movement of material occurs between and along the fascial layers of skeletal muscle [30, 40, 69-71]. The associated data implies that the material will be in contact with perimysium/epimysium layers *in vivo*. Thus, a valuable knowledge base can already be established; the fascial layers of the skeletal muscle have been studied in some detail and their composition understood [26, 72, 73]. This established knowledge aids in selecting potential factors to model *in vitro* that could affect *in vivo* drug fate following an IM injection.

In order to effectively model conditions and events observed *in vivo* in an *in vitro* setting, we first consider the fundamental physiological properties of skeletal muscle that may influence the fate of the injected material. These include temperature, pH, and pressures within the tissue. We then consider tissue elements present at the IM injection site; ECM components will be particularly important as the distribution of an IM injected formulation appears to be primarily in the perimysium/epimysium fascial planes. While these factors can impact the fate

of a drug delivered by IM in a formation that is intended for rapid release, they could also impact the formation of a depot intended for long-acting, extended release of a drug.

1. Temperature

Skeletal muscle temperature ranges between 34 and 37 degrees Celsius (°C), and this heat contributes to the maintenance of homeostatic core temperature in man [74, 75]. Depending on the drug formulation in question, storage temperature recommendations for IM injectables are often set at refrigeration (4-8 °C) or ambient (15-30 °C) temperatures, as set out in US Pharmacopoeia guidelines [76]. Some pharmaceuticals like vaccines are stored at, and could be consequently dosed at, low temperatures [77]. Thus, a drug formulation will likely experience a temperature transition following an IM injection. Healthcare professionals could theoretically "pre-warm" injectables to physiological temperature prior to administration, but this is not likely to happen commonly in real-world applications. It is possible that exposure of a drug formulation intended for IM injection to such temperature changes could alter protein-binding properties and increase motility, affecting drug fate at the injection site [78, 79]. Macromolecules, in particular, can undergo aggregation as a consequence of such a temperature transition [80-82]. While the majority of published data relating to protein aggregation are in the context of macromolecule formulations and *in vivo* fate-testing, reports of prolonged exposure to temperatures outside of storage recommendations prior to dosing have described negative impacts on drug stability and, consequently, efficacy [80, 83-86]. Outside of actual therapeutics, liposome vehicles in LAI injectables have the potential to aggregate, potentially impacting/altering the intended extended-duration drug release profile [87]. Reports regarding IM drug aggregation in any form are scarce, yet such events should not be disregarded. Simple, economical assays using biologically-informed fluids at physiological temperatures could predict aggregation due to temperature *in vivo*, as determined by size-exclusion high-performance liquid chromatography for example [80]. This can be especially useful, when considering that aggregation assay data from formulation development may not represent events at physiological temperature. Aggregation behaviours can be non-Arrhenius in behaviour, and thereby mathematically complex to extrapolate to physiological temperature [88, 89]. Aside from the potential to induce aggregation, temperature can directly affect therapeutic success for injectables reliant on polyester based technologies for sustained release, such as poly(D,L-lactic-co-glycolic acid) (PLGA) materials. In the case of long-acting risperidone (formulated

with PLGA microparticles), such *in vitro* testing revealed a dramatic temperature-dependent change in release rate [90]. Upon a temperature shift from 37°C to 45°C, there was an approximate 6.5 times increase in risperidone release from the PLGA microparticles. Though not necessarily a drug-specific drug fate event, *in vitro* assessment of temperature-related drug-release events can inform decisions regarding specific injection site location for drug administration. These assays can relate to increase in temperature in a specific injection site during, for example, inflammation or fever, or indeed temperature differences between two discreet injection environments (i.e. SC and IM regions, where SC temperature is approximately 34°C versus 37°C in IM sites).

2. pH

Human skeletal muscle pH can become slightly acidified following intensive exercise, moving from homeostasis of 7.4 to approximately 7.2 [91]. It has been historically reported that tissue pH can drop as far 5.4 under inflammatory conditions [92]. Interstitial fluid (ISF) can become acidified for extended periods of time; acidified ISF has been observed in the pathology of type II diabetes [93]. This is due to the "lack" of inherent buffering components in the ISF, such as albumin with respect to blood plasma [93]. Although, even under such exertive conditions, the bicarbonate-based buffer system of the body appears to maintain a relatively constant pH [94]. Thus, this consistent pH range of skeletal muscle will dictate conditions experienced by an IM injection, driving the injection site environment rapidly back to homeostatic pH where some drugs may be sufficiently insoluble to the extent of affecting their release into the systemic circulation [95]. When homeostatic pH is excessively disturbed, however, the effects of acidosis/alkalosis are widespread. Oxygen transport, the cardiovascular and nervous systems, and metabolism of tissues (including skeletal muscle) are all affected [96, 97]. Thus, if the pH of the drug depot establishes a durable acidification or alkalinization, this could affect the viability of skeletal muscle at an IM injection site. Furthermore, changes in the pH of blood plasma can negatively impact protein-binding of the API, potentially affecting PK outcomes [79].

The buffering capacity of the ISF itself is primarily drawn from bicarbonate buffering, though albumin also contributes some buffering capacity [98]. Total extracellular fluid bicarbonate in the human body has been reported to be approximately 350 mmol [99]. It has been proposed that roughly 24% of total body weight is extracellular fluid, while ISF accounts for around 20% total body weight [100]. Assuming a 70 kg human and a fluid mass of 1 g/mL, one can calculate

the concentration of bicarbonate in the interstitial fluid to be ~17.3 mM. This is in general agreement with a reported range of 5-54 mM from mathematical calculation and/or sampling of blood serum [101].

Beyond the bicarbonate buffering of the IM injection site, poly-charged ECM and serum components provide an additional layer of buffering capacity [102]. Collagens, which are net positively charged, and hyaluronic acid, which is net negatively charged, represent two of the most abundant ECM elements [103-106]. One of the most common serum elements is albumin, which has been suggested to play an important buffering role [98]. Albumin is present in the interstitial fluid at ~0.19 mM [102]. Thus, there are multiple and extensive mechanisms present at the IM injection site to rapidly recover homeostatic pH following the introduction of a drug formulation that could neutralize potential electrostatic interactions between a drug substance and formulation components that might occur at a non-physiological pH.

Due to the multiple and extensive mechanisms at the IM injection site to stabilize physiological pH, drug and formulation components will rapidly move toward a pH state where dissolution/diffusion from the injection site could be dominated by interactions with ECM elements that would not be anticipated at the pH of the formulation. With such knowledge in mind, IM pH thusly becomes a parameter of formulation design. The acidic, poorly water-soluble diazepam is formulated with propylene glycol and acidic compounds for buffering and solubility, creating an injectable with acidic properties [95, 107]. At the opposite end of the scale, water-soluble fosphenytoin is formulated with tromethamine (used as an acidosis therapy in itself) in alkaline TRIS buffer adjusted to pH 8.3-9.3 by titration with hydrochloric acid or sodium hydroxide as required [108, 109]. Aqueous preparations for IM injection are generally recommended to be within a pH range of 2-12 [62, 110], but the extent of formulation buffering capacity may be more important than the starting pH. For wholly oil-based formulations, pH becomes a less pressing concern, as pH relates to concentrations of protons and hydroxide ions in aqueous solutions that are not in abundance in such material.

In certain cases, a change in pH is sufficient to catalyse aggregation events for pharmaceuticals. As an IM-specific example, salmon calcitonin can aggregate as fibrils at neutral pH [111-114]. Reports for other macromolecules and small molecules, however, appear to be scarce. Despite this limited range of knowledge, such events could nevertheless occur at physiological pH (essentially neutral) in combination with temperature changes.

With this initial understanding of *in vivo* pH effects, it is therefore logical to suggest that physiological buffering agents can therefore influence potential aggregation events. Whilst the bulk of buffering in interstitial fluid is through the carbonate/bicarbonate axis, soluble proteins like albumin, which can act as a buffer, could be involved. Buffers have the potential to negatively impact crucial characteristics of macromolecules by, for example, lowering the melting temperature and altering physical conformation [115, 116].

3. Pressure

Skeletal muscle presents a densely packed, tightly arranged environment. Injected material must adapt to the physical space and disperse between fibres and fascicles, as opposed to distributing within individual cells [40, 71]. This results in pressure introduced into the tissue that is directly related to the injected volume. Depending upon formulation characteristics such as viscosity and hydrophobicity, the material could hypothetically spread through the tissue or form a more defined depot with limited spreading (as would be the case for LAIs) [29, 67, 69, 117]. Pressures arising from muscle movements could impact the degree of this spreading. The "IM fluid pressure" of resting "thigh muscles" in man has been reported to be between -3 and +10 mmHg [118, 119]. Contraction can dramatically increase this pressure to above 1000 mmHg in the vastus medialis [120]. It can be readily hypothesised that contracting muscle tissue could force further spreading of an injected formulation between fascicles and away from the injection site. This would theoretically expose the injected material to an increased surface area of IM components and vasculature, influencing the rate of drug absorption.

Hydrostatic and colloid osmotic pressures could further influence the dissolution/diffusion of IM injected material. Hydrostatic pressures, in theory, would be disrupted during the injection process. After material has been deposited however, the hydrostatic pressure should then resolve to a homeostatic state. As such, it could be colloid osmotic pressure that influences the rate at which interstitial fluid components interact with the formulation to possibly affect drug uptake into the systemic circulation [119]. In situations where muscle "swelling" can occur, such as during exercise, this may further affect the interstitial fluid volume that can interact with the injected formulation and, therefore, affect dissolution/diffusion rate [121]. Furthermore, exercising muscles will experience an increased blood flow rate to meet metabolic requirements [122]. As such, movement of interstitial fluid (formed from

extravasated blood plasma) through exercising skeletal muscle will be elevated, and this has been found to increase the absorption rate of IM-injected atropine [123, 124].

4. Extracellular matrix (ECM)

Following an IM injection, drug and formulation components will come into contact with the well-vascularized ECM network of the fascicular fascia prior to their uptake into systemic circulation. Potential interactions between a drug and/or formulation component could realistically alter their release rates from the IM injection site. The degree to which contact occurs could be dependent on the volume of injectable administered. Larger injected volumes could spread further through the tissue and, hypothetically, come into contact with a greater surface area of ECM compared to a smaller injected volume. The ECM has been described as an "undulating layered system", maintaining orderly structure of the muscle tissue during movement [28]. This is the result of the array of proteins that combine their individual properties to create a structural lattice that is more than the sum of its parts. Depending on the specific region of ECM that is of interest, the distribution and proportion of components can vary. For example, collagen IV is commonly observed in significant quantities in the "basement membrane" of ECM in contact with cells, whilst relatively little is observed in fascial layers where collagens I and III are dominant instead [26, 72, 125, 126]. As it can be hypothesised with reasonable confidence that IM-injected pharmaceuticals interact with perimysium and epimysium layers *in vivo*, the primary components of these fascial layers will be focussed on [40, 69, 127].

4.1 Collagen

Collagens form the bedrock of the ECM and account for between 10-13mg/g of "wet tissue" [128, 129]. These proteins are formed of trimers arranged in a triple helix [130]. Collagens can be found as homo or heterotrimers: collagen I is a heterotrimer of type I alpha-1 and alpha-2 chains ($[\alpha\text{-}1(I)]_2$ and $[\alpha\text{-}2(1)]_2$), whilst collagen III is a homotrimer of three type I alpha-3 chains ($[\alpha\text{-}1(III)]_3$) [130]. Collagens have a unique motif of three amino acids known as the "collagenous domain" of Gly-Xaa-Yaa, where Xaa and Yaa are typically proline and hydroxyproline, respectively [131, 132]. The small glycine permits the helical arrangement of collagen fibrils [130]. Collagens are either fibrillar or non-fibrillar. The fibrillar variety includes the ubiquitously expressed collagens I and III (dominant features of the skeletal muscle ECM) which form strong, robust structures due to continuous repeats of the collagenous domain.

These are not only crucial for maintaining the structure of muscle, but for supporting vasculature, lymphatic vessels and nerves that traverse skeletal muscle tissue [126, 133]. Non fibrillar collagens, such as collagen IV, have frequent interruptions in the repeats of the collagenous domain for enhanced flexibility compared to fibrillar collagens [130]. The collagens cooperate with themselves and other ECM components to form extended networks which provide structural integrity and functional ability of skeletal muscle. The collagens are positively charged entities at homeostatic pH and could interact with injected material through electrostatic interactions [103, 104]. Collagen binding can be specifically exploited to enhance local drug activity if necessary [134-137].

4.2 Hyaluronic acid

Hyaluronic acid (also known as hyaluronan; HYA) has been described as a "biological lubricant", reducing internal friction during muscle contraction because of its viscoelastic properties and osmotic attraction of water whilst stabilising the ECM in its distribution within connective tissues [138-140]. It is a negatively charged polysaccharide of no fixed molecular weight: the length of the polysaccharide chain determines the overall molecular weight [106, 138]. HYA chains can form extended, "entangled" networks which, depending on the molecular weight and size of the network, can dictate the permeability of molecules [140]. In the rat, HYA content of skeletal muscle has been reported to be between 0.09 and 0.13mg/g of wet tissue, with equivalent tissue distribution to that observed in man [128, 139, 141]. It has been located between muscle fibres (in man) as well as throughout the perimysium and epimysium (in the rat) [139, 140].

4.3 Interstitial Fluid

The interstitial fluid (ISF) is extracellular fluid that bathes cells and tissues, accounting for approximately 20% of total body weight [142]. ISF volume in muscle ranges between 70 and 120 µL/g of wet tissue in the rat [128, 129]. Interstitial fluid turnover is continuous, as blood plasma extravasates into the interstitial space at arterial regions of intramuscular vasculature and returns to the vasculature at venous regions as according to colloid and hydrostatic pressures [143]. The composition is influenced by extravasated blood plasma and consists of a variety of soluble proteins and ions [102, 142, 144-148]. Generally speaking, the ion composition of ISF is analogous to blood plasma; sodium and chloride ions are dominant electrolytes in extracellular fluid alongside a smaller proportion of other anions and cations

[149]. The relative content of proteins such as lipoproteins and albumin (common protein components of the ISF) is lower in ISF than plasma [144, 150]. ISF serves to distribute cell-signalling molecules and nutrients whilst removing metabolites from the tissue [93]. As the components of the ISF influence the tonicity of the ISF itself, the tonicity of the injected material can influence the degree of interaction with ISF. That is, hypertonic injectables could cause ISF accumulation around the deposited material [151]. This, therefore, could influence the extent of interaction with this solvent and dissolution/diffusion of the formulation/API from the injection site.

Current *in vitro* technologies

Presently, there are no established *in vitro* systems modelling *in vivo* outcomes of the IM injection site. Such a system would expedite testing of novel pharmaceuticals through reliable prediction of *in vivo* outcomes that then informs the design of *in vivo* and clinical trials. SC-specific modelling systems discussed here were designed around parameters that can affect the fate of SC injectables. It is possible that this approach could also be used to generate an IM-specific *in vitro* system. There could be further benefit if a tractable system could be established where specific IM environment parameters could be considered and altered to better understand specific interactions or events.

When developing *in vitro* fate-testing systems, the key is to effectively model the injection environment of the target organ. When considering the SC and IM parenteral routes, this means reproducing the ECM, and the plethora of components found there. To this end, two approaches can be taken: cellular or acellular. Where cellular systems use living tissue/cell cultures, acellular systems use protein-based designs. Both approaches model ECM components and their matrices with unique capabilities and caveats. SC as well as IM models are discussed here to highlight existing SC systems, and how the approaches used could be adapted for IM use. Firstly, we consider cellular *in vitro* systems, which are typically *ex vivo* tissues or organs, or “micro-tissues” derived from cell cultures.

Genoskin (MA, USA) have described a commercial *ex vivo* SC injection model termed HypoSkin using human tissue donated by patients undergoing abdominal surgeries [152]. The model consists of the three major layers of the skin: the epidermis, dermis and subcutaneous space. This is preserved and maintained within a “gel-like matrix” under “standard cell culture

conditions" [152]. Hyposkin® has been described in assessing local reactions such as inflammation following SC injection of endogenous and bacterial pro-inflammatory molecules [152]. Such an approach represents a truly biorelevant model for studying injections by using living human tissue (including host immune cells) and its ECM to generate biorelevant predictions of *in vivo* outcomes.

Meanwhile, Afshar and colleagues described the *in vitro* generation of human-derived skeletal muscle "micro-tissues" grown in 96-well plates for applications including drug discovery and pre-clinical toxicity assessments [153]. This approach benefits from using viable, human-derived skeletal muscle tissue and ECM for predicting drug fate of pharmaceuticals in a variety of formulations. The three-dimensional (3D) cultures described are invaluable in being both biologically and physiologically similar to their *in vivo* counterparts. This would yield superior, biorelevant data compared to two-dimensional (2D) cultures, which allow limited movement of applied/injected material through the tissue and ECM network. The authors do not define the lifespan of the "micro-muscles", but data measurement over a 14-day window implies a minimum of two weeks. The authors propose that "long-term culture" of these tissues is possible.

Napaporn *et al*. described an *in vitro* model using whole extensor digitorum longus muscles from rats [154]. These whole-muscle models were applied in assessing myotoxicity of buffers (in a pH range of 2 – 6) over a 2-hour time course. Whole isolated muscles offer the most biorelevant model of skeletal muscle possible, hypothetically including local immune cells to help identify local inflammatory events. Studying myotoxicity of injected "formulations" in this format is ideal, as such events would indeed occur within a few hours post-IM injection (i.e. whilst the excised muscle organ is still viable).

We now consider acellular approaches, typically utilising some form of hydrogel to represent the *in vivo* injection site. Leung *et al*. described a simple *in vitro* system to model diffusion of pharmaceuticals for formulation optimisation [155]. After noting how the ECM of the SC and IM injection sites behave as a gel, the system was based around agarose hydrogels formed in phosphate buffered saline (PBS) [155]. Briefly, a drug-loaded agarose hydrogel was formed in the bottom of a quartz cuvette. Another agarose gel, without drug, was then formed on top of the drug-loaded gel. The remainder of the cuvette was then filled with PBS. This model supposedly predicted *in vivo* release of SC insulin and naproxen, to demonstrate application in testing biopharmaceuticals as well as small molecules.

Prestwich *et al*. developed a 3D ECM-mimetic gel, amenable to 3D cell culture, for use in applications including drug discovery and toxicology [156]. This gel was developed using covalent cross-linkage via thiols added onto ECM components such as hyaluronic acid and glycosaminoglycans. Such models can be utilised to identify ECM-drug interactions post "injection", allowing formulation optimisation to either a) evade ECM interactions or b) exploit ECM-specific binding characteristics of the drug.

In a similar tack, Narayanan *et al*. developed a 3D ECM-mimetic gel, amenable to cell culture, for "biomaterials-based skeletal muscle regeneration" [157]. Cross-linkage of HA, chrondroitin sulfate and PEG-3400 was achieved by addition of thiol groups and click-chemistry. The benefits of such a gel are similar to those of Prestwich *et al*., namely identifying drug-ECM interactions after depositing formulations within the matrix.

Wassenaar *et al*. decellularised porcine muscle tissue to access and solubilise the ECM components in acidic conditions. Hydrogels are then generated after subjecting the solubilised ECM to physiological pH and temperature. Thus, these hydrogels are composed of the acid-soluble components of the ECM (including collagens). These are ideal biorelevant, acellular versions of skeletal muscle, having been derived from skeletal muscle tissue itself. This model, in principle, is optimum for assessing *in vitro* drug fate and potential drug-ECM interactions post-injection. The authors tested this concept by studying ECM-binding of cobinamide, and reported their *in vitro* data correlated well to previous *in vivo* datasets.

Kinnunen *et al*. described an acellular *in vitro* tool that can predict some aspects of *in vivo* outcomes for SC injected pharmaceuticals, named the ***S***ub***c***utaneous ***I***njection ***S***ite ***S***imulat***or***, abbreviated to Scissor (Pion) [158]. The Scissor models "critical parameters" of the SC injection site, which were appraised in a previous article by this group (and found to be independent of the cellular components) [159]. The authors described a hyaluronic acid hydrogel contained in a semi porous cuvette, submerged within a physiologically informed bicarbonate-based buffer. The composition of the gel is representative of the SC environment, and the gel-like properties of the ECM are accounted for (as done by Leung *et al*.). The entire system is maintained at homeostatic temperature and pH. Studies were conducted to illustrate how human bioavailability of IgG1-based monoclonal antibodies could be supposedly predicted with this tool [158, 160]. Theoretically, this system could be used to study both short-acting and LAI pharmaceuticals, provided that sterility and the homeostatic conditions can be maintained.

In vitro modelling of tissues and/or organs aims to carefully represent the tissue of interest whilst tightly controlling parameters for consistency between experiments. To represent the tissues, actual tissue can be extracted from living organisms, generated *de novo* from cell lines or represented in an acellular fashion. Ultimately, the choice of approach determines the duration and nature of experiments that can be conducted. This includes amenability to high-throughput assessments and/or answering specific and detailed questions. With cellular systems, especially in the case of whole extracted tissues (such as whole muscles), the viability can be limited to several hours or several days. Therefore, the methodologies described by Napaporn and Genoskin are limited to short-term studies e.g. ECM-drug interactions and fate of aqueous-formulation, fast-acting pharmaceuticals (such as epinephrine and antibiotics) [152, 154]. Despite this disadvantage, cellular systems are the only choice for studying/observing myotoxicity, inflammatory events and tolerability of formulations in *in vitro* settings, which may have effects on drug fate. In order to measure cell-based responses and reactions, the system must be cellular. Furthermore, detailed questions regarding the ECM and the fate of injected materials could indeed be studied with appropriate pharmaceuticals, even in this short time frame of model viability. Yet the costs, technical expertise and time associated with generating and using tissue-based approaches would imply limited usability for high-throughput studies.

The *de novo* and acellular methods generate tissues/models as required, and *de novo* tissues can theoretically be maintained for as long as necessary for experiments. Acellular models could be stored for indefinite periods of time prior to experimentation, offering flexibility to researchers whilst being relatively simple to implement, as tissue viability is irrelevant in these cases. Therefore, such approaches as described by Afshar, Leung, Wassenaar, Prestwich and Kinnunen could study drug fate of both “fast”-acting pharmaceuticals and LAI pharmaceuticals (where assays might last beyond 7 days at a time for LAI studies) and for addressing detailed questions of, for example, interactions of pharmaceuticals with specific ECM components [153, 155, 156, 158, 161]. Furthermore, the cellular method of Afhsar *et al*. can, to a certain extent, address myotoxicity, inflammatory events and tolerability of formulations as *ex vivo* approaches can. These studies in *de novo* or acellular settings could be conducted in single experiments or upscaled into high-throughput capacity, particularly in cases such as the 96-well plate format of Afshat *et al*.

However, with acellular systems, the ECM must be modelled in a biological manner (i.e. containing actual ECM components) to be biorelevant. For example, the Leung method uses

agarose-based hydrogels which are not representative of the ECM proteins observed in SC/IM injection sites, disregarding the possible interactions of pharmaceuticals with ECM proteins and their networks. Similarly, Prestwich's hydrogels were formed from gelatin (i.e. hydrolysed collagen) as opposed to collagen, which may not be truly representative of collagen matrices *in vivo*. Furthermore, these hydrogels were chemically crosslinked using thiols. Such non-physiological cross linkage methods must be used carefully so that excessive linkage does not occur, and that residual chemical mediators after the reaction do not interfere with pharmaceuticals introduced to the system.

Using non-physiological buffers to model tissue fluids requires careful consideration too: tissue fluids are bicarbonate-based, so PBS would not accurately model these fluids (as used by Leung *et al*.) [155, 159]. The solubility of hydrophobic pharmaceuticals in such buffers should be considered also; incorporating lipophilic components such as lipoproteins and albumin into the buffer system (which Kinnunen *et al*. omitted from their buffer design) could be critical for effective modelling of drug fate. This relatively simple consideration would boost the application of the utilised system to a wider variety of pharmaceuticals and their formulations.

A central, critical factor that determines the success of a model is its consistency. Is the model reproducible and, as such, reliable? Any model must be well defined and characterised to achieve this consistency. For whole muscles, this would be somewhat complicated. Which muscle should be used, from which organism, and how do you swiftly assess the muscle prior to experimentation? *De novo* tissues by comparison would be simpler to a certain degree, as cell culture conditions (and therefore tissue growth) can be very tightly controlled. Nevertheless, assessments must be made of the ECM between preparations. Would histology be ample, or would individual ECM components need to be quantified? Acellular systems, especially those specifically designed to be tractable, should be the most consistent in theory. However, acellular systems derived from decellularisation of tissues (such as those described by Wassenaar *et al*.) have similar caveats as extracted tissues. For example, is the ECM of the same muscle from two different rats equal in terms of composition? Are there significant differences in collagen type I concentration for example? Hydrogels derived from ECM components (either by tractable design or from decellularised tissue) could be tested for consistency by rheology. Assessment of the storage and loss moduli, subsequent Tan(δ)

values and shear thinning would indicate whether individual hydrogels of a given methodology are consistent between each other.

Finally, the choice of cellular or acellular approach influences the ethical impact of the model itself. The acellular and *de novo* tissue methods are in better agreement with the "3 Rs" approach to pharmaceutical research, by reducing and replacing *in vivo* animal models [162, 163]. The use of extracted tissues for cellular or decellularised models introduces significant ethical and financial implications as a result of their origin. Such long-term implications must be considered from the beginning when developing technologies to model *in vivo* events.

Such *in vitro* approaches can emulate specific aspects of the injection site in a tractable manner to predict *in vivo* drug fate, such as excessively slow dissolution/diffusion outcomes [164]. This vital information could allow for mitigation strategies to be implemented during formulation development and re-testing *in vitro* before entering *in vivo* trials. The significance of such data, if it is truly representative of *in vivo* outcomes, cannot be overstated. Currently, for example, there are no predictive pre-clinical models for injection of biopharmaceuticals [152, 165]. Relying on animal models, which are ultimately not representative of outcomes observed in man, is a dangerous game; *in vivo* data must be manipulated to inform clinical trial dose sizes [162, 166-169]. Without reliable predictions of drug fate and bioavailability, underdosing in a Phase I study is an expensive mistake and overdosing can put individuals needlessly at risk. Thus, having some form of *in vitro* predictive technology is critical for the efficient and safe development of pharmaceuticals being administered by injection [158, 160, 170]. Furthermore, such tools will ultimately reduce and replace a large number of animals used in biomedical research, thereby adhering to the "3 Rs" of animal use in research and improving the ethical impact of scientific endeavours [162, 163].

Complexities of Assessing IM Injectables

A wide range of drugs are currently administered by IM injection, including hydrophilic and hydrophobic small molecules as well as peptides, proteins, and complex agents such as vaccine antigens. Critically, the spectrum of formulations that are used for these IM injections vary between aqueous liquids and oils as well as solutions and particulates, with the properties of these materials resulting in rapid or durable release profiles. For example, drugs formulated with oils generally exhibit controlled, extended release, such as in the cases of

testosterone cypionate, which uses cottonseed oil, and haloperidol decanoate, which uses sesame oil [62]. Thus, when one considers drug fate at an IM injection site, it is likely that distinct IM injection site parameters will be critical for different types of drug formulations being administered. Additionally, the time course of minutes to hours for drug release from the site could be most relevant for an aqueous formulation of a hydrophilic small molecule, while hours to days might be more relevant for an oily formulation of a hydrophobic drug. Release of some drugs could require conversion of a pro-drug form, that might occur spontaneously or through local enzymatic actions. LAIs are typically intended to release over months and some vaccines are not really intended to leave but rather draw immune cells to the injection site. Thus, development of a universally applicable *in vitro* IM injection site model would be technically challenging, and likely to be prohibitively expensive. **Table 1** tabulates information regarding the vehicle, the release technologies and release profiles employed in FDA-approved IM drugs. In terms of extended-release technologies, pharmaceuticals can be delivered as prodrugs and/or associated with particulate delivery technologies. Macromolecules do not strictly require such considerations, though these agents may be chemically modified to enhance prolonged action *in vivo*.

IM Injection Site Parameter Prioritisation

When considering IM drug fate, the type (i.e. small molecule versus macromolecule) and size of pharmaceutical can influence the nature of interactions with the IM environment. In our considerations discussed here, it must be acknowledged that literature regarding macromolecules is greatly limited at present, and the hypotheses herein are presented with reasonable consideration.

An ideal scenario with *in vitro* model development would permit the development of a single entity with which multiple scenarios/tissues could be modelled accurately for drug fate testing of both small molecules and macromolecules. However, such a scenario is highly unlikely, as the various parenteral delivery routes used in medicine all greatly differ from each other in physical conformation and composition. The IV route, for example, is solely fluid with no physical matrices for injectables to interact with and diffuse through. The SC and IM sites contain relatively small fluid volumes that disperse throughout extended matrices of physical material. Although there are similarities in composition, there are distinct differences between the properties of SC and IM environment components that significantly challenge

690 the development of a "universal" *in vitro* tool for studying *in vivo* drug fate, such as the
691 relative concentration of collagens and HYA.
692
693 When identifying critical parameters for modelling the IM environment, it could be said that
694 the most fundamental components in the IM environment are collagens I and III, which are
695 the dominant collagens in the fascicular fascia [26, 72]. There is a sound body of evidence
696 (derived primarily from rat models) proposing that IM injected material moves along the
697 inter-fascial planes of skeletal muscle, distributing along what could be described as a path of
698 least resistance within the skeletal muscle organ [40]. Magnetic resonance imaging (MRI) of
699 oil depots revealed how the shape of IM depots *in vivo* depends on the arrangement of
700 fascicles, moving longitudinally along the fascial planes [71]. Furthermore, inter-fascial
701 movement was observed by MRI whilst determining PLGA microparticle fate [171]. Similarly,
702 LAI depots of cabotegravir were observed to disperse along fascial planes [40]. Analogous
703 observations were made with fluorescent microspheres used to study the IM movement of
704 interstitial fluid and VEGF has been shown to distribute along perimysium layers post-IM
705 injection [30, 70]. Darville *et al*. observed paliperidone-palmitate distribution along
706 epimysium *and* perimysium boundaries from a confined central depot [172]. It is reasonable,
707 therefore, to take the collagen-rich composition of these fascial layers as a truly critical
708 parameter for a novel *in vitro* IM model.
709
710 The collagen-rich fascial layers are relatively thick, being typically 150-200 µm in depth but
711 with reports of up to 0.55 mm in the erector spinae [73, 173]. The perimysium layer of skeletal
712 muscle appears to be a criss-cross matrix of larger collagen fibres, supplemented by thinner
713 collagen fibrils [174, 175]. This arrangement of the collagen fibres, as well as the density of
714 the matrix, could dictate drug diffusion through the network [174, 176]. Similar to collagens,
715 HYA-based networks can contribute to steric hindrance of drug release from an injection site
716 [140]. The significance of HYA for *in vivo* dissolution/diffusion and, consequently, *in vivo* fate
717 of high molecular-weight biopharmaceuticals (i.e. monoclonal antibodies) from the SC
718 injection site has been described [158, 160]. In combination with collagen networks, HYA-
719 based networks are, therefore, of interest for the IM-modelling system. Both sites contain
720 HYA, yet the relative proportion is higher in SC regions than IM sites [128, 141, 177]. Such
721 knowledge was applied in the creation of the SCISSOR tool, where the hydrogel at the core of
722 the system is chiefly formed of hyaluronic acid at high concentrations (>1mg/mL) [158]. HYA

723 is present at the IM site as a biological lubricant to minimise friction during flexing of the
724 skeletal muscle elements. Whilst a detailed range of IM-HYA concentrations has not yet been
725 determined, it is reasonable to propose that the concentrations of fibrillar collagens would
726 far exceed that of HYA; it is the fibrillar collagens that determine and maintain the physical
727 conformation of skeletal muscle for effective function, not HYA.
728
729 Based on such ruminations, one can rationally propose that an IM modelling hydrogel would
730 rely upon a collagen I/III type environment, whilst SC modelling would depend on HYA and
731 collagen IV, for example. Interstitial fluid is a common element that unites these two
732 environments, but even then, considerations must be made how to model this fluid
733 effectively and efficiently. Using a bicarbonate/carbonate system, for example, would be
734 relatively complex by requiring continuous pH monitoring/supply of CO_2 gas into the medium.
735 Whilst this is the most biorelevant system to use, the question ultimately lies in whether such
736 a complicated system should be used. There is potential for alternative buffering molecules
737 to be used alongside the expected mixture of salts observed *in vivo*, such surgical fluid-
738 replacement solutions for example.
739 A "universal" *in vitro* tool that can model SC and IM drug fate may not be realistic or feasible
740 to generate. However, it is reasonable to propose that, with suitable modifications, one
741 generic format could be used for modelling a variety of injection sites. For example, a SCISSOR
742 system "skeleton" could be used to accommodate cartridges of various
743 components/compositions to model various injection sites with appropriate physiological
744 fluid buffers. These cartridges could represent the vitreous humor of the eye, or the SC and
745 IM sites to name a few examples.
746
747 Concerns related to restricted release from an IM injection site due to steric hindrance may
748 be particularly relevant for very large agents such as modified adenovirus vectors, where
749 collagen matrices were overcome with co-administration of collagenase [178, 179]. While it
750 is conceded that these studies are not IM specific, observations regarding steric hindrance
751 are noteworthy and should be recognised for their worth in developing a novel IM modelling
752 system. A variety of small molecule drugs have been formulated as microparticles,
753 microspheres, vesicles, and micelles to achieve certain PK profiles, though not all of these
754 approaches are utilised for IM formulations [61, 180-184]. Such vehicles can be relatively

large, ranging from nanometres to micrometres in diameter that could hypothetically influence their dissolution/diffusion *in vivo* [61, 185].

Using an environment derived from collagen I/III and HYA could provide insight into the extended-release profiles of such formulations where steric hinderance could be of concern. While steric hindrance represents a mostly non-specific effect of striated muscle ECM elements on drug fate following an IM injection, specific interactions between the drug and these same ECM elements is also possible. Targeting pharmaceuticals to bind components of the ECM is not a novel concept, such approaches have been utilised in attempting targeted drug delivery in cancer therapies by exploiting collagen binding domains [134-137]. In some instances, it is intended that injected pharmaceutical should interact with IM ECM elements to limit distribution post-injection. Vascular endothelial growth factor (VEGF) has N-terminal domains dedicated to interacting with proteoglycans, for example. Understanding of IM VEGF drug fate *in vivo* has been described previously and identified extensive association of VEGF with perimysium ECM matrices prior to engaging with myocyte cell surface receptors [30]. In a similar fashion, IM botulinum neurotoxin type A (BoNT/A) was observed to remain at the injection site (with some inter-fascial distribution, albeit limited) [186]. Such drug fate characteristics are governed by the intrinsic nature of the drug: proteins such as VEGF and BoNT/A inherently localise at the injection site, including distributing through the tissue regions cored by needle insertion [30]. Not all IM-macromolecules will exhibit these characteristics. Indeed, there is a confounding lack of data and understanding of how injected proteins distribute within skeletal muscle tissue after IM injection. As such, one can only hypothesise as to the interactions that may be occurring, and thereby governing drug fate. Nevertheless, it is of great value to understand the interactions and movements of therapeutic proteins such as VEGF and BoNT/A. This is especially so when considering how LAI small molecule drugs have been identified distributing along fascial planes and their ECM matrices [40].

Specific interactions between a drug and ECM components could be affected by the ISF through its actions to return the IM injection site to homeostatic conditions. Most IM injected drugs are formulated at non-physiological conditions with regard to pH and ionic composition. Thus, there will be a dynamic exchange between formulation components and ISF elements that will occur at the IM injection site over a time frame that will be generally

788 dictated by the injected volume. Distribution differences of a set volume could affect the
789 surface area generated, which might further affect this rate of exchange. As this dynamic
790 exchange causes the IM environment to move from that of the formulation to homeostatic
791 conditions, there is the potential for interactions between the drug and ECM components to
792 be enhanced or reduced. Thus, the ISF can play an important role in drug fate in the IM
793 injection site, so accurate modelling of the ISF is crucial to generating a realistic *in vitro* model.
794 The use of physiologically informed buffer solutions to develop any *in vitro* model of the IM
795 injection site would align with consideration for interactions between drugs and ECM
796 components [187-189]. Previously, such an approach was taken by Kinnunen *et al*., using an
797 ISF-style buffer containing ions and carbonate continuously buffered with carbon dioxide gas
798 [158]. While such an approach seems reasonable, it is certainly possible to build upon that
799 system with the introduction of even more elements of the ISF. An alternative approach
800 would be to use fluid-replacement solutions, such as those used in surgery, as a starting point
801 [149]. Hartmann's solution that uses sodium lactate/lactic acid for buffering, whilst containing
802 many ions found in ISF and in similar proportions [149]. Such materials could serve as a
803 starting material for generating a novel ISF buffer solution. Due to its lack of volatility
804 compared to the bicarbonate-based buffer system, a lactate/lactic acid buffer may be more
805 useful under conditions of extended *in vitro* studies.
806
807 **The Future of In Vitro IM Injection Site Modelling**
808 Any novel *in vitro* system for predicting drug fate must be biorelevant but should ideally avoid
809 being over-complicated, which would confound costs, user-friendliness and limit the ultimate
810 value and use of the system. A delicate balance between simplicity and complexity must be
811 struck to create any *in vitro* system. Where cellular *in vitro* systems provide comprehensive
812 environments representative of the IM site, there longevity and costs can be prohibitive
813 (especially when using whole excised tissues from rat models for example). Acellular systems
814 exploit easy-to-use hydrogels, but with a simplified, specific composition of several ECM
815 proteins disregarding the plethora of components *in vivo*. The choice of cellular versus
816 acellular could depend on the pharmaceutical in question. This then makes the development
817 of a "one size fits all" *in vitro* tool particularly challenging, especially when such a system
818 should ideally model outcomes for both small molecules *and* macromolecules.
819 The ECM is a vastly complex and heterogenous environment, though some elements are
820 potentially playing a more significant role than others in specific instances. Appropriate *in*

vitro assessment of fast-acting versus sustained release formulations represents an additional challenge to the identification of an *in vitro* model where sufficient sterility will be required for LAI experiments. Simplification of the IM injection site to certain ECM components, as proposed here, could permit a system design where the core modelling components can be a) readily stored for long periods of time and b) applied for extended experiment time frames. Furthermore, such core modelling components would be readily tractable, and could be fine-tuned in a controlled manner as required for specific needs and experimental objectives.

The literature has reported excellent progress in modelling drug fate at the SC injection site, with benefits and caveats specific to each example. However, it must be said that the SC models do not perfectly fit into the IM-modelling challenge. The physical nature of the SC and IM sites are not equal (e.g. in temperature). The same is true in their composition; although similar in terms of components present (e.g. Col1 and HYA), the actual quantity of those components differs vastly between the two environments. As such, the SC and IM sites are separate entities and should not be treated as identical to each other. Despite these differences, the approaches and logic used to develop these SC systems could be employed in modelling the IM site.

Overall, methods and knowledge appear to be already present in the published literature that provide the basis for one or more *in vitro* models that could be used to better define drug fate at an IM injection site. Our understanding of a) skeletal muscle fascial components, b) distribution of IM injected material *in vivo*, and c) improved clarity of the specific questions that could be asked in a tractable model of the IM injection site, provide the basis for the generation of *in vitro* IM-specific models. These models could be used to better understand the fate of current IM injected drugs and potentially predict *in vivo* performance of new drugs and formulation approaches. Such *in vitro* systems are likely to save time, costs, and reduce the potential to put patients at risk during clinical testing.

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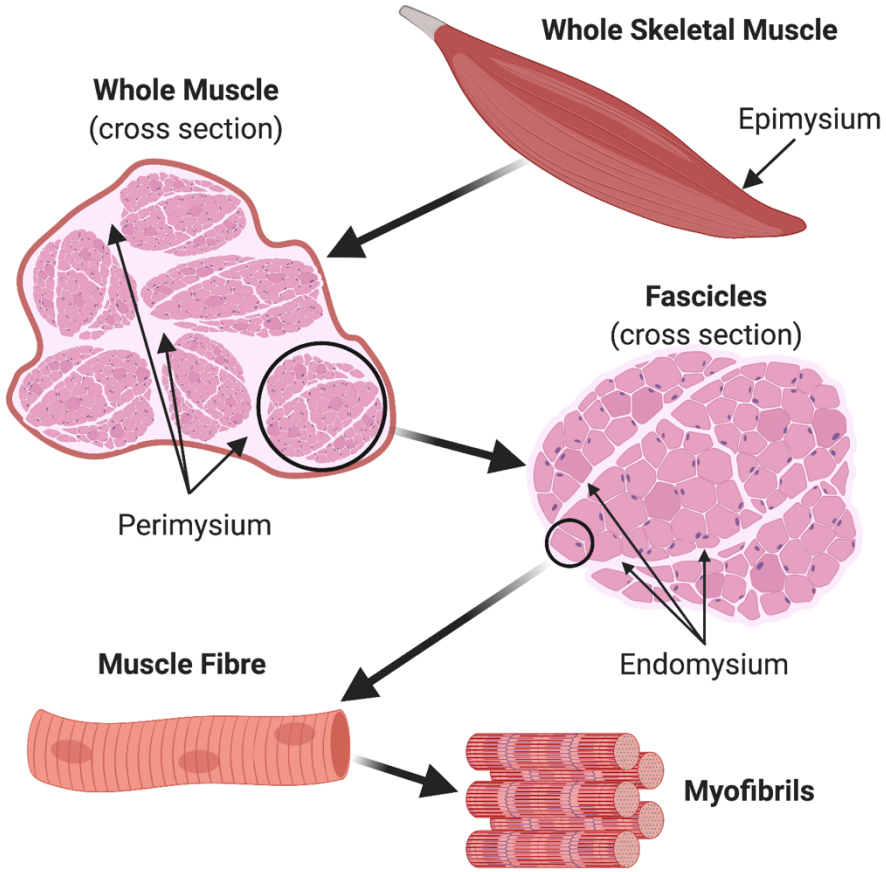

Figure 1 - Physiology of the skeletal muscle organ. The extracellular matrix surrounds and supports the major functional elements. The endomysium supports muscle fibres, the perimysium supports whole fascicles, whilst the epimysium supports the entire organ itself.
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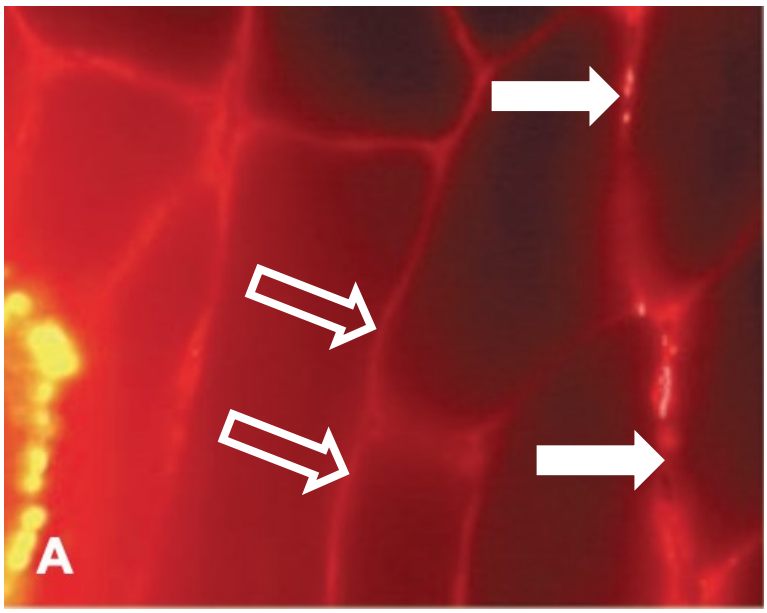

Figure 2 – Intramuscular associations of VEGF after 2 hours in IM injected rat biceps. Fluorescence microscopy revealed how VEGF (labelled with "Texas red") associates with the ECM (open arrows) and vasculature (closed arrows) of the skeletal muscle tissue. Reproduced from [25] with permission.

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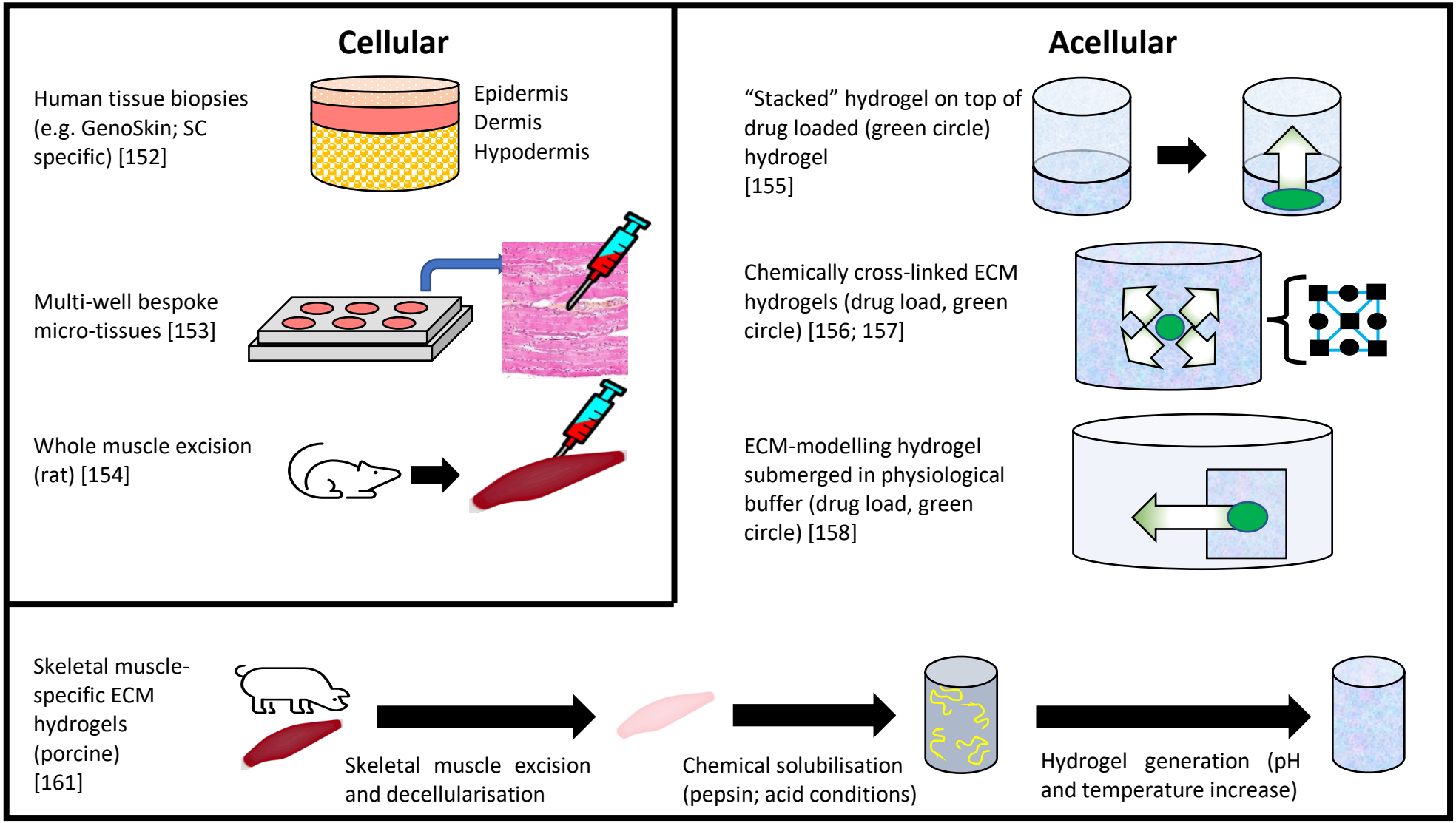

Figure 3 – **Summary of *in vitro* technologies studying drug fate.**

***Table 1* – Current FDA approved IM-injectable pharmaceuticals with known pharmacokinetic data available from the *PharmaPendium* database (Elsevier).**

Short-Acting				Long Acting			
Drug Name	**Vehicle[1]**	**Molecule Type[2] & MW[3]**	**Release Technology[4]**	**Drug Name**	**Vehicle[1]**	**Molecule Type[2] & MW[3]**	**Release Technology[4]**
Adrenaline (Epinephrine)	AQ	SM; VL	N/A	Aripiprazole	AQ	SM; Low	MP [190]
Amikacin Sulphate	AQ	SM; Low	N/A	Aripiprazole Lauroxil	AQ	SM; Low	MP, PD [190]
Ampicillin Sodium/Sulbactam Sodium	AQ	SM; Low	N/A	Botulinum Toxin Type A	AQ	Protein; V.H.	N/A
Asparaginase	AQ	Protein; VH	PEG [191, 192]	Buprenorphine	AQ	SM; V.L.	MP [193]
Asparaginase *Erwinia chrysanthemi*	AQ	Protein; VH	N/A	Buprenorphine Hydrochloride	AQ	SM; V.L.	MP [193]
Atropine	AQ	SM; VL	N/A	Fulvestrant	Oil	SM; Low	N/A
Atropine Sulphate	AQ	SM; VL	N/A	Haloperidol Decanoate	Oil	SM; Low	PD [194]
Atropine/Pralidoxime Chloride	AQ	SM; VL	N/A	Hydroxyprogesterone Caproate	Oil	SM; V.L.	N/A
Aztreonam	AQ	SM; VL	N/A	Interferon β-1a	AQ	Protein; Large	N/A
Bleomycin Sulphate	AQ	SM; Med	N/A	Leuprolide Acetate	AQ	Peptide; Med	MP [195]
Butorphanol Tartrate	AQ	SM; VL	N/A	Medroxyprogesterone Acetate	AQ	SM; V.L.	MP [196]
Calcitonin, Salmon	AQ	Peptide; Med	N/A	Methotrexate Sodium	AQ	SM; V.L.	N/A
Capreomycin Sulphate	AQ	SM; Med	N/A	Methylprednisolone Acetate	AQ	SM; V.L.	PD [197]
Cefepime Hydrochloride	AQ	SM; Low	N/A	Naltrexone	AQ	SM; V.L.	MP [198]
Cefotaxime Sodium	AQ	SM; VL	N/A	Octreotide Acetate	AQ	Peptide; Med	MP [199]
Cefotetan Disodium	AQ	SM; Low	N/A	Olanzapine Pamoate	AQ	SM; Low	MP, PD [200]
Cefoxitin Sodium	AQ	SM; VL	N/A	Paliperidone Pamoate	AQ	SM; Low	PD [56]
Ceftazidime	AQ	SM; Low	N/A	Palivizumab	AQ	Protein; V.H.	N/A
Ceftriaxone Sodium	AQ	SM; Low	N/A	Pasireotide Pamoate	AQ	Peptide; Med	MP, PD [201]
Cefuroxime Sodium	AQ	SM; VL	N/A	Pegaspargase	AQ	Protein; High	PEG [2]
Clindamycin Phosphate	AQ	SM; Low	N/A	Risperidone	AQ	SM; V.L.	MP [202]
Cyanocobalamin	AQ	SM; Med	N/A	Testosterone Cypionate	Oil	SM; V.L.	PD [203]
Diazepam	AQ	SM; VL	N/A	Testosterone Undecanoate	Oil	SM; V.L.	PD [204]
Diclofenac Potassium	AQ	SM; VL	N/A	Triptorelin Pamoate	AQ	Peptide; V.L.	MP [205]
Dihydroergotamine Mesylate	AQ	SM; Low	N/A				
Ertapenem Sodium	AQ	SM; VL	N/A				
Fosphenytoin Sodium	AQ	SM; VL	N/A				
Gentamicin Sulphate	AQ	SM; VL	N/A				
Glucagon Hydrochloride Recombinant	AQ	Peptide; Med	N/A				
Glycopyrrolate	AQ	SM; VL	N/A				
Interferon α-2a Recombinant	AQ	Protein; Large	N/A				
Interferon α-2b Recombinant	AQ	Protein; Large	N/A				
Ketorolac Tromethamine	AQ	SM; Low	N/A				
Leucovorin Calcium	AQ	SM; Low	N/A				
Lorazepam	AQ	SM; VL	N/A				
Methylergonovine Maleate	AQ	SM; VL	N/A				
Midazolam Hydrochloride	AQ	SM; VL	N/A				
Morphine Sulphate	AQ	SM; VL	N/A				
Nalbuphine Hydrochloride	AQ	SM; VL	N/A				
Naloxone Hydrochloride	AQ	SM; VL	N/A				
Ondansetron Hydrochloride	AQ	SM; VL	N/A				
Pegademase (Bovine)	AQ	Protein; Large	PEG [192]				
Pentamidine Isethionate	AQ	SM; Low	N/A				
Piperacillin Sodium	AQ	SM; Low	N/A				
Pralidoxime Chloride	AQ	SM; VL	N/A				
Ranitidine Hydrochloride	AQ	SM; VL	N/A				
Tazobactam Sodium	AQ	SM; VL	N/A				
Thyrotropin α	AQ	Protein; Large	N/A				
Tobramycin	AQ	SM; VL	N/A				
Tobramycin Sulphate	AQ	SM; VL	N/A				
Triamcinolone Acetonide	AQ	SM; VL	N/A				
Trimethobenzamide Hydrochloride	AQ	SM; VL	N/A				
Ziprasidone Mesylate	AQ	SM; Low	N/A				

Short-Acting: Time between doses is < 1 week.

Long-Acting: Time between doses is ≥ 1 week.

Vehicle: Aqueous (AQ) or Oil-based formulation.

M**olecule Type**: Type of pharmaceutical.
Peptide (<50 amino acids).
Protein (≥50 amino acids).
Small molecule (SM).

Molecular Weight (MW)
Very Low (V.L.; <500 Da).
Low (<1 kDa).
Medium (Med; <5 kDa).
High (>10 kDa).
Very High (V.H.; >100 kDa).

Release Technology: Mechanism employed for extended drug release/activity.
Microparticles (MP).
N/A (not applicable).
PEGylation (PEG; drug conjugated with polyethylene glycol polymer chains).
Prodrug (PD).

Data for this table was collected from:
[1] rxlist.com
[2, 3] pubchem.ncbi.nlm.nih.gov

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Table 2 – Summary of attributes and critical components that could affect IM drug outcomes

Parameter	Properties/Effects	Potential drug fate impact
Temperature	Temperature "shock" upon injection; inflammation or fever	Drug aggregation; enhanced LAI release rate
pH	Acid/alkaline formulation transition to homeostatic pH. Transition of drug from oily deposit to buffered IM environment	Chemical and physical drug stability; drug aggregation
Hydrostatic/Colloid osmotic Pressure	Transient increase in local pressure within muscle tissue; tissue "swelling" and accumulation of interstitial fluid	Diffusion limitation; diffusion acceleration
Collagen	Interactions with anionic entities, extended interwoven matrices	Steric hindrance; drug binding (net positive electrostatic charge)
Hyaluronic acid	Interactions with cationic entities, matrices	Steric hindrance; drug binding (negative electrostatic charge)
Interstitial fluid	Rate of fluid movement/accumulation respective to pressures; incorporation of lipophilic drugs into lipoproteins	Diffusion kinetics; tissue swelling; transporting lipophilic drug cargo

Adapted from [159]