Published in final edited form as:

J Pharm Sci. 2018 May ; 107(5): 1247–1260. doi:10.1016/j.xphs.2018.01.007.

Challenges and opportunities for the subcutaneous delivery of therapeutic proteins

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Abstract

Biotherapeutics are a rapidly growing drug class and well over 200 biotherapeutics have already obtained approval, with about fifty of these being approved in 2015 and 2016 alone ¹. Several hundred protein therapeutic products are still in the pipeline, including interesting new approaches to treatment. Due to patients' convenience of at home administration and reduced number of hospital visits as well as the reduction in treatment costs, subcutaneous (SC) administration of biologics is of increasing interest. While several avenues for treatment utilizing biotherapeutics are being explored, there is still a sufficient gap in knowledge regarding the interplay of formulation conditions, immunogenicity, and pharmacokinetics (PK) of the absorption of these compounds when they are given SC. This review seeks to highlight the major concerns and important factors governing this route of administration, and suggest a holistic approach for effective SC delivery.

Keywords

absorption; bioavailability; biotechnology; immune response; formulation; pharmacokinetics; physicochemical properties; proteins

2. INTRODUCTION

Recently, a shift toward safe and effective SC delivered proteins has garnered the attention of major pharmaceutical companies and health care providers alike. The biggest advantage to this route of administration is a cheaper, more convenient method of dosing that accommodates both patient and physician. Patient convenience likely improves due to ease of at home self-administration, as many of these therapeutics require multiple doses for long term therapy ². Even for therapeutics that must be administered by a physician, SC injection take mere minutes compared to the one to several hours necessary for intravenous (IV) infusion ^{3,4}. In addition to these general advantages, unique advantages associated with individual therapeutics have been observed on a case by case basis. Erythropoietin is an

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often cited example of a therapeutic protein with better efficacy dosed SC rather than IV, due to a prolongation of systemic exposure when given this route that allows for a reduction in dosing ^{5,6}. This phenomenon can be described by the concept of flip-flop kinetics, where the absorption rate is the limiting step for drug clearance ⁷. Flip-flop kinetics have also been reported for low dose interferon beta-1a in monkeys, with an absorption rate constant of 0.104 h⁻¹ compared to an elimination rate of 0.2 h⁻¹, which has beneficial clinical implications for SC dosing ⁸. Another benefit has been documented for alemtuzumab, where evidence of improved tolerance by reducing or eliminating flu-like symptoms caused by IV infusion related reactions ⁹. A reduction in adverse injection related event severity has also been reported for rituximab in a clinical trial comparing the two routes of administration ¹⁰. While this route of administration offers significant practical benefit, it is not without complication. Many of the obstacles associated with SC delivery can be categorized based on three general concerns: formulation issues, immunogenicity, and PK (Figure 1, top). Table 1 highlights several examples of approved proteins and their given formulations, as well as a summary on their bioavailability, immunogenicity, and concentration. One such means to improve protein therapeutic success is to treat each of these characteristics as interdependent on one another throughout development, using a mix of biophysical studies and preclinical trials to guide a holistic approach.

SC dosing is generally dosed as a small volume comparative to IV infusions, which allow for large volumes and proper dispersal of protein. Higher volumes of injection generally lead to patient discomfort and sometimes pain at the site of administration ¹¹. Considering the EC50 of monoclonal antibodies (mAbs) is quite high, the dosing range is between 150 mg to 1.2 g per dose. The resulting concentrations generally lead to protein crowding, which has been shown to increase the risk of aggregation due to protein-protein interaction ¹². Therefore these formulations require added excipients and stabilizers that improve not only conformational stability but also colloidal stability of proteins within this crowded environment. Aggregation can interfere with protein absorption and allow increased interactions with immune cells within the SC space, as larger particles can remain trapped at the injection site for longer periods of time. There is still debate whether or not this environment is more or less immunogenic than traditional IV dosing, a phenomenon that garners a great deal of attention ¹³. Finally, PK variability has been seen for a variety of SC dosed proteins, particularly mAbs that even share similar structure and molecular weight. From incomplete and variable bioavailability ranging from 50 to 100% to differences in absorption rate (0.1 to 0.4 inverse days) and Tmax (2 to 8 days), it is clear that several factors are involved in determining the PK fate of antibodies dosed via this route ¹⁴. Problems associated with limited or incomplete bioavailability can be associated to unfavorable uptake, immunogenicity, poor absorption profiles, protein aggregation, and other factors.

3. ANATOMY AND PHYSIOLOGY OF THE SKIN

The skin is the largest organ in the body, and in addition to its physiological roles of protection, hydration, and thermoregulation it provides an excellent route for many pharmaceutical agents. The skin is composed of three important layers: the epidermis with sublayers stratum corneum, lucidum, granulosum, spinosum, and basale; the dermis; and the

hypodermis, otherwise known as subcutaneous tissue ¹⁵. The epidermis is primarily a protective layer containing keratinocytes and dendritic cells (Langerhan's Cells), which will later be discussed for their immunological role. This layer constantly rebuilds itself, constantly in motion allowing restructuring and building of the outermost exposed stratum corneum ¹⁶. Between the epidermis and dermis lies a nonrestrictive, porous barrier that allows fluid and cell exchange while supporting both layers with connective fibrils and collagen ¹⁷. Within this barrier and part of both of the aforementioned layers lie various sweat glands and hair follicles which aid in temperature regulation and protection from the environment. The upper dermis layer contains loose connective tissue and many blood capillaries, as well as nerve endings and pain receptors highly responsive to external stimuli ¹⁸. Deeper within the skin tissue nearing the border and transitioning into the subcutaneous space lies the remainder of the dermal layer and the extracellular matrix (ECM), which contains fibroblasts, macrophages, and adipocytes. At the junction between the dermis and hypodermis, larger blood vessels and lymphatic networks aid in fluid homeostasis and transportation of immune cells from the ECM and surrounding area to internal lymph nodes for immune processing ¹⁹. The hypodermis is located between the skin and the deep fascia covering the muscle tissue ²⁰. The deep subcutaneous tissue contains mostly adipocytes and connective tissue responsible for fat storage, internal organ protection, and insulation ¹⁶.

The physiological makeup of the ECM is a subject that has not been sufficiently explored for drug absorption, especially for protein pharmaceuticals that undergo specific uptake pathways and have an inherent risk for aggregation in environments they were not specifically designed to endure. A general depiction of the anatomy and physiology of the skin is presented in Figure 2 (top). There are several components of the ECM in the SC space with the potential to limit or improve absorption of macromolecules, and all of these components should be considered when designing a drug to be given via this route. The two major cell types that make up the ECM are the aforementioned fibroblasts and adipocytes, both with specific function. Fibroblasts are responsible for structural components such as collagen, elastin, and glycosaminoglycans, while adipocytes specialize in energy storage and makeup adipose tissue ^{21,22}. In addition, macrophage cells are present in the same environment and serve as a host defense against foreign antigens prior to their advancement into the general circulation. The ECM consists mainly of adipose tissue connected by a fiber network of connective tissue septa, which links the dermis to the deep fascia ²³. This fibrous network is made up of collagen, glycosaminoglycans, and elastin to provide structure and necessary flexibility/rigidity necessary to segregate tissue and regulate intracellular transport and communication.

Collagen is the main structural protein in the ECM and is the most abundant protein in mammals ²⁴. Collagen networks in the SC space in particular are made up of elongated fibrils of Type 1 collagen, and in addition to providing structure and support is the key component of skin tissue that benefits all stages of wound healing, including aiding the closing of wounds. As seen in Figure 2, the triple helix structure of collagen is stabilized by many hydrogen bonds and form elongated, well ordered fibrils that are extremely durable. This collagen network is positively charged at physiological pH, although this charge may be less relevant due to an overall negative charge in the ECM. As stated before, collagen fibers link the dermis to the deep fascia, and coupled with elastin provide structure and

elasticity to the ECM. Elastin, as its name implies, is an elastic protein found in tissue that holds its shape even after the tissue is stretched or flexed ²⁵. Composed of elastin fibers and fibrillin, elastin serves a specific function of restoring shape to skin after it is agitated, and when coupled with the collagen network provides structure and elasticity within the ECM. Other components that aid in creating and maintaining this fibrous network include fibronectins and laminins crucial in the network organization and mediation of cell direction and function ²⁶.

Glycosaminoglycans are another integral part of the ECM, a major component of synovial tissue and fluid made up of a polysaccharide chain with a repeating disaccharide unit consisting of an amino sugar and an uronic sugar ²⁷. As opposed to positively charged collagen, glycosaminoglycans are highly polar and negatively charged, which contributes to the negative charge on the outside of cells. They serve to attract water and are viscoelastic, adopting conformations that strengthen the ECM to withstand high compressive forces ²⁸. One important function of glycosaminoglycans, especially hyaluronic acid which is abundant in the SC space, is regulation of transport of substances and cells across barriers. Hyaluronic acid controls interstitial fluid content and viscosity, as well as the hydraulic conductivity of the ECM ^{21,25}. Being negatively charged, it stabilizes the positively charged collagen fiber network while giving the entire matrix strength and fluid support. As is the case with collagen, glycosaminoglycans such as hyaluronic acid function to repair tissue in the event of wound damage, making them critical components in skin tissue.

This overview of skin anatomy and physiology should highlight the function and importance of the cells and components found within this space. Of particular importance to the SC administration of therapeutic antibodies is the interplay between the ECM environment with protein characteristics, which can be distributed into three major interdependent categories: potential for immunogenicity, absorption attributes governing PK, and formulation considerations. Protein immune response and the risk factors associated with SC injection and its impact on drug absorption will be considered first, as it is a widely contested area in the biotherapeutic industry.

4. IMMUNOGENICITY CONSIDERATIONS

One of the most important treatment related factors that contribute to immunogenicity is the route of administration and the development of anti-drug antibodies (ADA) ²⁹. Much of the current understanding of immunologic response stems from the use of vaccines, in which an external substance is injected via the SC route in the presence of an adjuvant to elicit an immune response from the host ³⁰. Vaccination studies have illustrated a more efficient and effective immunological response against antigens administered SC over IV ³¹. While this traditional immune response is typical for antigen based vaccines, there is still a gap of knowledge surrounding the immune response triggered by antibodies that have been specifically engineered to mimic human proteins such as human mAbs and endogenous replacement products that do not contain these adjuvants ^{13,32}. This argument starts to unravel however with specific examples of SC dosed therapeutics with immune response nearly identical to IV treatment. Abatacept is one such example, and has been shown in clinical trials to have low immunogenicity no different than IV with and without

methotrexate use when dosed SC^{33,34}. The anatomy of the SC space has been widely debated as to its immunologic potential. It may be argued that because the SC layer contains few dendritic cells, potent antigen presenting cells and primary initiators of T-cell response, injection directly into this layer circumvents potential immunogenicity^{35,36}. However, elegant studies using peptide-MHC complex of fluorescently labeled antigen coupled with CD40 tracking revealed the role of cutaneous dendritic cells in eliciting immune response; specifically Langerhans cells in the epidermis and dermis resident dendritic cells³⁷. While traditional immunogenicity mediated clearance can occur as ADAs develop over time, degradation and clearance from the site of injection by immune cells is important. These antigen presenting cells migrate to the SC space upon injection, process the administered protein, and present it to T-cells present in the lymphatics as a “first-pass” immune interaction prior to reaching systemic circulation (Figure 2, bottom). This has been reviewed and explained thoroughly elsewhere, specifically by Germain and colleagues^{13,38,39}. Thus, the migratory potential of these cutaneous dendritic cells drives immunogenicity of SC administered proteins.

4.1. Immune response to therapeutic proteins

ADAs may impact the pharmacokinetics of these therapeutics by altering clearance, but also effect the safety and efficacy by severely or completely diminishing pharmacological activity^{40,41}. Antibodies against therapeutics contribute to rapid elimination of compounds that would otherwise have a long half-life, such as is the case with mAbs^{42,43}. The bottom of Figure 1 represents a simulated PK profile for a model mAb dosed IV or SC at 1 mg/kg. It signifies the interplay between protein absorption, clearance and the effect of immunogenicity on PK. Examples include adalimumab, where long term therapy suggests that anywhere from 18% to 38% of patients develop ADAs that inhibit therapy^{43,44}. Increased clearance has been associated with anti-adalimumab antibodies in a cohort of studies⁴³ and reduced serum trough levels have been linked to the presence of ADAs⁴⁵. Neutralizing antibodies (Nabs) may lead to a loss in efficacy by targeting the active site of the protein. Additionally, immune system effects such as anaphylactic response, inflammation at the site of injection, and “serum sickness” may occur⁴⁶. Immunogenicity risk has been defined as the probability of an ADA occurrence as related to its consequence⁴⁷. While these risk factors have been reviewed extensively elsewhere^{48,49}, they are generally categorized into treatment, patient, and product specific factors. For purposes of this review, only the product specific factors such as formulation variables and biophysical characteristics will be discussed in the context of SC absorption and immunogenicity.

The immune response mechanism within SC space highlighted earlier suggests that preventing the migration of cutaneous dendritic cells after SC administration of a therapeutic protein could be an effective strategy to mitigate immunogenicity. It has been established that there is a link existing between aggregates and immunogenicity because they are pro-inflammatory, although not all aggregates are immunogenic and oxidation of protein is a critical contributor.^{29,50–55} Due to the complex folding process of proteins, several types of aggregates with distinct molecular and biophysical characteristics have been identified^{56–58}. These aggregates are classified based on size, conformation, morphology, co-valent modification, and reversibility. FDA Guidance for Industry written in 2014 suggests

assessments be made for subvisible particles 2–10 microns in size be measured over the course of the product's shelf-life, as well as efforts to characterize 0.1 to 2 micron sized particles ⁴⁷. Carpenter and colleagues have shown that subvisible aggregates (between 0.1 μm and 100 μm) are more immunogenic ⁵¹. Characterizing aggregates of this size poses an analytical challenge, however there are several biophysical techniques emerging to address this issue ^{59,60}. For subvisible particles, techniques such as light obstruction, flow imaging, coulter counting, and nanosite technology has been highlighted and reviewed by Singh and Toler ⁶¹. The impact of conformation based aggregates on SC administered proteins is still emerging. We and others have shown that not all aggregates are immunogenic, in studies including Factor VIII (FVIII), IgG antibodies, and a model bispecific construct antibody ^{50,62}. In particular, one study involving native hFVIII or its aggregated forms dosed into hemophilia A mice produced significantly more ($p<0.05$) inhibitory titers for native-like aggregates compared to nonnative or regular hFVIII ⁶². In another study Swiss-Webster mice were immunized with native-like IgG₂ κ mAb/bispecific antibody construct or variable oligomeric aggregates formed at different incubated temperatures. The results of this study indicated that exposure to a low concentration of small aggregates lead to higher immunogenicity when the animals were later rechallenged with intact native protein ⁵⁰. Retention of the biologic within this space also has the potential to trigger the innate immune response leading to degradation and elimination via macrophages ^{63,64}.

4.2 *In vitro* and animal models of immunogenicity

Predicting immune responses within the SC space has been exceedingly difficult, as it varies from protein to protein and doesn't depend on specific formulation considerations. Several *in vitro* and *ex vivo* systems have been developed to predict immune response within systemic circulation ^{65,66}, however these systems do not translate to the physiological nature of the SC space or reflect proper mechanisms as the protein transitions from injection site to lymph vessels and onward to the blood compartment. There are a variety of three-dimensional human skin models that are generally created to replicate individual skin characteristics, however these approaches are too simplistic for reproducing full immune responses ⁶⁷. One example includes full thickness models with incorporated Langerhan's cells in the epidermis where upon antigen exposure actively relocated to a dermal compartment incorporating fibroblasts ⁶⁸. These models have the disadvantage of utilizing collagen obtained from non-human species, and therefore have difficulty in mimicking the structure and denseness of the human dermis. This can lead to different absorption and distribution *in vitro* compared with human pharmacokinetics. While this model fails to predict immune processing further downstream, it represents an important first step towards an *in vitro* recreation of the immune system within the skin. In a recent review, Rosenberg highlighted immunogenicity assessments that occur during drug development ⁶⁹. These include *in silico* models to predict peptide-HLA-class-II affinity epitopes on proteins, a common epitope that triggers CD4+ T-cell response, *in vitro* methods of peptide-MHC-II affinity, and *in vitro* assays replicating T-cell epitope identification as well antigen processing and presentation ^{70–73}. While each of these prediction approaches generate immune risk potential, as is pointed out in the review a universal risk ranking system that incorporates all potential outcomes has not been implemented ⁶⁹.

Potential immune response is difficult to predict at the preclinical setting, as most conventional animal models drastically overestimate immunogenicity in humans and hence are poor predictors of potential immune responses⁷⁴. Few studies demonstrating a link between animal immune responses to that in man^{75,76}. Brinks offers a detailed accord in the use of animals to predict human immune response, in which she enforces that they are in need of critical evaluation⁷⁷. Overall, preclinical models such as these allow observation of relative immunogenic potential and mechanistic studies otherwise not ethical in human trials. Conventional small animals including mice and rats develop a classical immune response against recombinant human therapeutic proteins. However, instances where mouse models have shown to produce an immune response similar to humans exist, including a hemophilia A mouse model that mimics the conditions present in patients with an absence in Factor VIII⁷⁸. Because the underlying disease state in severe hemophilia A is the complete absence of protein and FVIII shares conserved regions between species, the immune response developed is considered a classical response that can be mimicked through these mouse models^{73,79,80}. Further, it has been shown that the cell types that drive the immune response in humans and mice tend to be common in these species, suggesting some preclinical value for this species^{81,82}. In addition, the humoral immune response is qualitatively similar between mice and humans. For mAbs especially, the anti-idiotypic antibody response (the immune response against the variable region of the protein) generated in mice models offers some translational value to human ADA response. Humans, like mice, are not tolerant to these variable regions. For proteins conserved across species, non-human primates hold some predictive value due to similarities to the human immune system, primarily cytokine signatures and ADA production⁸³. Immunogenicity of recombinant human growth hormone dosed in rhesus monkeys, as an example, was shown to be similar to that seen in humans clinically⁷⁵. For proteins not conserved or less conserved between species, transgenic mice have been used in the past as predictive models^{84,85}. Mice developed are immune-tolerant to the administered protein, so immune responses generated for these animals are representative of the same breaking of tolerance seen for human proteins that develop an immune response. Minipigs have also begun seeing use as an alternative to rodents due to skin and SC homology to humans, however these animals differ significantly in antigen presentation via major histocompatibility complex haplotypes as well as lymphatic structure and are not appropriate for direct comparison to human⁷⁶. While animal models certainly retain value for prediction of relative immune response to therapeutic proteins, the value of these preclinical models is still limited.

5. PHARMACOKINETIC CONSIDERATIONS

The pharmacokinetic considerations of SC administered therapeutic proteins requires understanding the fate of the biotherapeutic in the context of SC anatomy and absorption. Generally, the SC space has few blood vessels, mostly including small capillaries that are slow to absorb small molecule drugs and practically impervious to larger molecular weight biotherapeutics. Once a therapeutic protein is injected into the hypodermis and interacts with the ECM, their fate is generally dictated by molecular factors such as size, charge, and uptake transporter affinity (such as FcRn for mAbs). For larger molecular weight proteins (>16 kDa), lymphatic uptake from the interstitial space plays a key role in eventual

absorption into systemic circulation. Many proteins boast favorable PK compared to small molecule drugs, with mAbs in particular boasting a half-life up to 4 weeks^{86,87}. This, coupled with the reduced systemic toxicity due to high target specificity, makes IgG based therapeutics a very important class of biologics.

5.1. Lymphatic absorption

For small molecule drugs and peptides with low MW, the tight junctions present in blood capillaries within the SC space can be overtaken, and lymphatic uptake is not necessary in the absorption process. However, lymphatic uptake is a primary contributor to the absorption of larger molecular weight protein therapeutics⁸⁸. The lymphatic vascular system begins with lymphatic capillaries present in a plexus at the dermal/hypodermal junction. From this plexus, the lymph drains into larger lymphatic vessels that pass through the fibrous network of the hypodermis⁸⁹. From these larger “trunk” vessels, lymph fluid and everything it carries enters lymphatic collector sites that run through the hypodermis to the first draining lymph node⁹⁰. Efferent, draining lymph vessels leave local nodes and transport lymph fluid into larger collecting lymphatic vessels. Finally, fluid is transported into the thoracic and other lymph ducts and onward to systemic circulation⁹¹. While this is generally the fate of larger MW proteins, there is still frequent debate as to the correlation between MW and percentage of lymphatic uptake in the absorption of therapeutic proteins^{91,92}.

The mechanism to which biotherapeutics are absorbed via the lymphatic system has been well characterized and described^{88,92,93}. In opposition to the small blood capillaries located in the hypodermis, lymphatic capillaries are open ended. Their endothelial cells have no tight junctions and overlap similar to shingles on a roof, which is how larger MW proteins can be taken up and transported away from the SC space⁹⁴. Lymphatic endothelial cells are attached to collagen and elastin fibrous networks by anchoring filaments which controls fluid uptake by the lymphatic system¹⁹. When interstitial pressure in the ECM is at equilibrium with pressure in the lymph, lymphatic capillaries and intercellular clefts are collapsed and no fluid uptake occurs^{19,95}. When interstitial pressure increases, for example when a hyperosmotic volume of fluid enters the ECM, these clefts are opened by the anchoring filaments attached to the fibrous network. Opening of these clefts allows influx of interstitial fluid and solutes such as large MW proteins into the lymphatics as pressure homeostasis is restored²⁰. The driving forces for the interstitial and lymphatic flows are the hydrostatic and osmotic differences that occur among blood, interstitium, and the lymphatics⁹⁵. Many other factors can impact lymphatic flow, including ECM composition, cell density in the SC space, blood pressure, rate of tissue metabolism, hydration, excessive adipose tissue as is seen in obese subjects, and exercise.

There have been few published reports detailing the lymphatic absorption of mAbs. One study exploring lymphatic uptake of trastuzumab in lymph cannulated rats found that 27% of the dose was recovered within 30 hours via the lymphatic system, which is actually an underestimation as the absorption process was likely not complete by that time⁹⁶. This number is probably closer to 50% as suggested by a compartmental PK model of first-order absorption into the peripheral lymph⁹⁷. Between 5 and 30% uptake into the lymphatics was observed by our own research group when studying the effects of osmolarity on rituximab

bioavailability in mice through lymph node excision and modeling estimation⁹⁵. Lymphatic absorption has also been studied in thoracic duct-cannulated rabbits for an IgG fusion protein lenercept, where recovery in lymph over 48 hours translated to 25–40% of the total dose⁹⁸.

5.2. Biophysical factors governing SC absorption

Much controversy still exists over the impact of certain biophysical characteristics of protein drug molecules have on their pharmacokinetics, and there is limited knowledge at all on how these parameters might affect absorption processes for drugs given SC. Factors such as molecular weight, surface charge, isoelectric point, and FcRn binding capacity are important in determining how and to what extent biologic agents are absorbed into the body, however with the exception of molecular weight most of these properties have not been explored in detail. Not only do therapeutic proteins have different functions and mechanisms of action, the wide differences in size, structure, and formulation factors make classification difficult or impossible when trying to describe absorption from an extravascular route such as SC. Each of these factors needs to be considered when designing biotherapeutics, as relying on an overarching absorption mechanism is inappropriate.

5.2.1. Molecular size—As mentioned previously, there are restrictions present within the SC space that limit the absorption of large MW biotherapeutics into the general circulation directly. To circumvent the tight junctions present along blood capillaries, proteins generally follow fluid uptake from the interstitium into the lymphatics. There are some discrepancies in the literature as to whether or not increasing molecular weight directly impacts the extent of lymphatic uptake and subsequent bioavailability^{20,92,99,100}. There is variability seen in the literature for a wide range of proteins with different molecular weights. First, animal model selection is likely a huge factor in determining SC absorption, since the physiological makeup of the lymphatics and the ECM can vary wildly from species to species^{20,91}. Although higher order species generally share absorption characteristics for larger proteins, small rodents such as rats or mice tend to be much more variable in lymph recovery⁹⁷. In one study SC injected doses of bovine insulin, bovine serum albumin, and erythropoietin reported extremely low lymphatic recovery (under 3%)⁹². A similar study in 1988 by Kojima and colleagues determined low lymphatic uptake of tumor necrosis factor that supplements this finding¹⁰¹. On the flip side, other research groups have concluded a larger lymphatic recovery upwards of 60–70% for pegylated erythropoietin in rats and only 20% in dogs, or the reverse of that (27% in rats and 73% in dogs) for another unnamed pegylated large peptide^{102,103}. These results are perhaps confounded by differences in injection site, formulation preparation, cannulation technique, or incomplete lymph sampling; or a physiological difference in rats compared to more consistent higher order species such as sheep or monkeys.

5.2.2. Molecular charge—One of the interesting topics for debate is the interaction of a protein's surface charge with the ECM, including charge at physiological pH and isoelectric point (pI). Surface charge of a biotherapeutic is a molecular property of its amino acid sequence and the pH of the surrounding solution, meaning that the behavior of the proteins can change wildly from its stable formulation state to the media within the ECM. This

property is complex, and leads to varying degrees of charge for the protein population in solution due to heterogeneity caused by deamination, isomerization, or post-translational modification^{104,105}. These factors are often uncontrollable in the manufacturing of protein therapeutics, and lead to acidic and basic variants of the original product that could have different absorption characteristics. Most therapeutics range in pI from 5 to 9, tending to remain close to neutral at physiological pH. While most mAbs have a pI somewhere around 6, some bear a slight positive charge (pI 7 to 9) at physiological pH^{105,106}. Some research has been done with protein charge and its interaction within the SC space, however the data does not offer a straightforward conclusion, and opinions are mixed. In one study, five proteins bearing positive charge from 20–78 kDa were analyzed for lymph transit time and it was found that these proteins were found in lymph at delayed times compared to negatively charged proteins within the same molecular weight range¹⁰⁷. In another study observing pharmacokinetic differences in charge variants of IgG1, no significant differences in AUC, Tmax, or Cmax were found; however the pI varied only slightly in this experiment (within 0.1 pI units) and may not have been sufficiently different to produce a noticeable difference in absorption and drug exposure¹⁰⁵. While charge variants of a protein do not typically make it to clinical trials, it is possible that pI variations exist and could explain differences in absorption. In one of few studies exploring the bioavailability of SC delivered mAbs with various isoelectric points, a minipig model was used to find correlations between clearance, pI, and SC bioavailability¹⁰⁸. While the pIs for the mAbs studied ranged only from 8.5 to 9.5, a slight correlation between pI and SC bioavailability was observed, with increasing pIs showing a small trend in reducing bioavailability. In a review by Boswell et al., there were mixed pharmacokinetic outcomes found when a protein was anionized or cationized including increases in clearance for both alterations and an increase in volume of distribution (V_d) for cationized species¹⁰⁹. However, this review notably does not offer any information as to changes in absorption or bioavailability when charge is altered on a protein administered via the SC route. Through our own research we have observed that increasing pI from 5.6 to 8.8 has profound impact on the overall pharmacokinetic profile, including absorption rate, bioavailability, and clearance driven by immunogenicity (unpublished data). While much is known on the effect of charge on tissue distribution and clearance, little is currently understood in regards to the effect charge alteration has on the rate and extent of absorption. The events that unfold within the SC space may hold the key in identifying aggregation potential, bioavailability, and other important pharmacological outcomes.

Region specific charge modifications may have the most impact on pharmacokinetic outcome. One research group found that altering an anti-Lymphotoxin α antibody or an undisclosed human antibody, essentially two different model mAbs, produced significant differences in not only observable concentration time profiles but also important pharmacokinetic parameters such as clearance, AUC, and interestingly bioavailability¹¹⁰. These proteins were modified to have +3 or -4 (anti-LT α) and +5 or -4 (HumAb4D5-8) charge compared to their wild type hosts. For anti-LT α , a +3 charge resulted in a drop to 31.4% bioavailability compared to 59.9%, while a -4 charge increased it to 69.9%. While absorption rates were not significantly different between species, Cmax and AUC changed dramatically, with the -4 variant having the highest drug exposure and lowest clearance.

Similar results were seen in the model human antibody, however in this case noticeable changes to absorption rates were recorded, although deemed not significant.

5.2.3. FcRn binding affinity—Another possible improvement that can be done during protein development is altering affinity to the FcRn receptor, and this has been extensively reviewed elsewhere ^{111–113}. As an example, one study indicated that for three Fc variants of mAbs with varying binding affinity to FcRn there was a corresponding increase to SC bioavailability with increasing FcRn receptor affinity: low affinity at 41.8%, medium at 86.1%, and high at 94.7% ¹¹⁴. This study, done in mice using antibodies derived from mice, concluded that while small changes in FcRn affinity may not significantly alter bioavailability between species, having no FcRn affinity at all is detrimental to elimination half-life and drug exposure. While FcRn has been shown in multiple studies to have a significant influence on clearance and half-life of a protein containing an Fc component (which includes mAbs, bispecific antibodies, Fc-fusion technology, and others), there are conflicting reports of how FcRn binding affects SC bioavailability. Datta-Mannan and colleagues found that no clear effect was observed on SC bioavailability in cynomolgus monkeys in a study including five different mAbs with varying FcRn affinity ¹¹⁵. It was concluded here that while differences were not observed in the study, multiple biopharmaceutical and physiological factors are likely to influence SC bioavailability. In contrast, when pH dependent FcRn affinity was included in IgG antibody design it was found that affinity at physiological pH 7.4 and endosomal pH 6.0 were both important in determining pharmacokinetic outcome ¹¹⁶. Having high affinity towards FcRn at pH 7.4 but not at pH 6.0 actually reduced bioavailability, where the reverse increased it. While these studies did not explore SC absorption rates or extents, it is possible that FcRn interaction is important for this administration route because it has been shown to affect bioavailability. There has been at least one study devoted to exploring SC bioavailability regarding FcRn interaction, and it was reported that a IgG1 mAb was around 3 fold higher in naive mice compared to FcRn deficient mice ¹¹⁷. In addition, IgG methionine oxidation at various sites in the Fc region has been shown to decrease FcRn binding by 20%, which has obvious indications for the PK of mAb variants that undergo even small oxidative stress ⁵⁵. Engineering mAbs or Fc containing molecules with consideration to FcRn binding is a promising strategy that should be utilized to improve pharmacokinetics of these therapeutics.

5.3. Injection volume and site of injection

In preclinical models, even a low injection volume creates a large pressure difference in the SC space that does not translate well to human dosing. For example, a 100 μ L injection in a 20g mouse equates to roughly 350 mL for a 70 kg human; a volume that would be impossible to inject via this route. Unfortunately, rodents receive massive volumes in comparison which leads to lateral diffusion that could cause shear stress and alteration in the glycocalyx structure which alters ECM structure and permeability of the endothelial barrier ¹¹⁸. Although there is difficulty in translating injection volume in rodents to that in humans, one study has been done that explores differences in bioavailability dependent on injection volume of rituximab ¹¹⁹. Although results were not significant, increasing the injection volume by 4 fold when injected into the back, or 3 fold when injected into the footpad had small differences in C_{max}, a possible indicator of rate of absorption. However, extent of

absorption (bioavailability) did not show any differences with various injection volumes. One major result of this study, however, was that location of injection (footpad vs back vs abdomen) had noticeably different absorption profiles as well as large variations in C_{max} even though the bioavailability was unchanged. In addition to this study, several preclinical and clinical studies have demonstrated that site of injection for administered protein drugs can influence bioavailability and rate of absorption, however there has yet to be an agreed upon opinion as to which site is best. In sheep, three sites including interdigital, abdomen, and shoulder resulted in bioavailability differences of 106, 85, and 92% respectively as well as large differences in C_{max} and absorption profiles¹²⁰. On the clinical level, AUC was significantly higher for growth hormone injected into the abdomen compared to the thigh (528.9 vs 239.34 units respectively), and C_{max} was raised significantly to 103.2 from 41.8 mU/L¹²¹. In a phase I study of golimumab, three injection sites (arm, abdomen, and thigh) were studied to assess differences in pharmacokinetics¹²². Although a small increase in C_{max} was reported in the abdomen and thigh compared to the arm, AUC and bioavailability remained relatively unchanged which indicated that pharmacokinetics were statistically similar for all injection sites. Additional examples include insulin where an injection in the abdomen produced a greater pharmacodynamic effect than in the thigh, and erythropoietin which resulted in a longer half-life of absorption and longer mean residence time in the thigh vs abdomen^{123,124}. Insulin is one of the few proteins studied demonstrating a clear difference in drug effect from one anatomical site to another, which might be due to differences in dermal thickness¹²³. It is still unclear whether anatomical differences in skin makeup or differences in lymphatic networking are the reason to the observable differences in absorption for all proteins, however at least for some proteins particular injection sites have proved beneficial.

5.4. *In vitro* and *in vivo* SC absorption models

There are few reliable *in vitro* or *ex vivo* systems in use to describe the absorption process for therapeutic proteins. One system developed by Kinnunen and colleagues utilizes a novel approach to recreate the buffer conditions within the ECM environment¹²⁵. Nicknamed Scissor for Subcutaneous Injection Site SimulatOR, this system models the fate of injected biopharmaceuticals after injection by utilizing a cassette based injection chamber modified to contain hyaluronic acid and physiological buffer that allows proteins within specific formulations to be injected while monitoring movement. Important features of this system include relevant charge and osmolarity interactions, as was illustrated in the diffusion of various mAbs with a range of pI and buffer formulations. While this system is truly revolutionary, it fails to include interaction with cellular components present *in vivo*; specifically interactions with immune cells that should be considered in the absorption process. Another *ex vivo* model has been used to demonstrate drug retention within rat SC tissue based on antibody charge¹²⁶. This technique utilizes homogenized tissue excised from animals to explore charge and formulation influence on interactions within the SC space. While this approach is lacking for obvious translational reasons, the results of this study confirmed that electrostatic interactions within this tissue play an interactive role, specifically with positively charged therapeutic proteins.

Although many animal models have been discussed here, there is still a sufficient gap in prediction and scaling of SC absorption from preclinical to clinical studies. Skin composition differs between species, and varies wildly between furred and non-furred animals. Rodents and furred species have loose connective tissue allowing for bilateral diffusion of much larger volumes not easily translated to human¹²⁷. Rodent models are generally utilized for pharmacokinetic considerations partially due to their low cost, however careful consideration should be applied to physiological differences. Another more recent animal model is the pig, which due to morphological and physiological similarities in the skin make for particularly useful applications to absorption studies¹⁰⁸. Even this model is problematic however, as pigs have an inverted lymphatic system that is difficult to translate to humans as well as decreased skin vasculature^{128,129}. Porter and colleagues have reported differences in lymphatic uptake between animal models studied, and concluded that careful consideration be given to what type of model should be used depending on the data needed¹³⁰. Monkeys see regular use as preclinical models given their similar homology to humans, however for mAbs these animals display higher bioavailabilities and overestimate absorption¹³¹. While some come close, there exists no perfectly translatable animal model to predict SC absorption of therapeutic proteins humans.

6. FORMULATION CONSIDERATIONS

Within the industry, protein formulations are often optimized for protein stability and storage considerations while maintaining safety of the product. Although stabilization of proteins within solution is studied universally for existing and future biotherapeutics, there is a fundamental lack of study regarding this formulation impact and its effect on pharmacokinetics. High-concentration formulations are commonly manufactured with stability in mind, and therefore require a sophisticated approach to reduce aggregation and denaturation¹³². While biophysical studies are generally done for new formulations to assess longevity and stability, these *in vitro* methods do not necessarily predict potential alterations to *in vivo* absorption or aggregation after injection. This section will highlight several important considerations to protein formulation development as they relate to protein stability and SC absorption, specifically the effect of increased concentration, buffer constitution, and stabilizing excipients.

6.1. Protein concentration

As mentioned previously, SC formulations for therapeutic proteins such as mAbs require large concentrations within a restricted volume often developed at or below 2 mL. From a design perspective this leads to significant challenges in predicting stability considerations for new antibodies, including long-term storage, preservation of therapeutic effect, and prevention of aggregation prior to and upon dosing. Proteins have an inherent high molecular sensitivity to various stress conditions such as heat, processing shear stress, exposure to light, and changes in pH¹³³. In addition, increasing protein concentrations are thought to increase aggregation potential for proteins^{134,135}. Higher viscosity and solubility issues generally plague development teams when higher concentration is formulated¹³². These formulation problems associated with highly concentrated formulations are generally not tested alongside PK outcome and immunogenic potential, which greatly underrepresents

their true impact. From a quality control perspective stability is generally extrapolated from studies performed at low concentrations which may not properly reflect true stability ¹³⁶. Measurements such as conformational stability generally done using circular dichroism or fluorescence emission done at smaller concentrations to determine differences in melting temperature, for example, do not always adequately assess protein stability ¹². An Empirical Phase Diagram approach utilized by Middaugh and colleagues is useful for describing the relationship between dynamics and protein stability for a variety of formulation conditions including pH changes, ionic strength, and temperature fluctuation. This technique has been applied to a variety of IgG compounds as well as other therapeutic proteins ¹³⁷. There are very recent attempts at analyzing highly concentrated formulations using bioanalytical methods, however these methods are not used throughout industry ^{138,139}.

One additional measurement that can be used to describe protein colloidal stability, and can utilize high concentration formulations, is the second virial coefficient (A₂, or B₂₂) generated by light scattering techniques ¹⁴⁰. While typically this measurement is used to determine absolute molecular weights of proteins by measuring various concentrations, A₂ can be used to determine the nature and strength of potential protein-protein interactions. For example, IgGs have been shown to display different aggregation patterns and unfolding characteristics as a function of pH and ionic strength ¹⁴¹. Our research team has also recently found differences in A₂ based on isoelectric point differences in a series of mAbs with CDR region point mutations, where higher pI was associated with a more positive A₂ coefficient compared to a pI two units less (unpublished data). This difference in A₂ correlates with CD and steady-state fluorescence emission data that also illustrates aggregation potential for only the higher pI mAb. Most interestingly, the biophysical results found in the study also correlated to differences in pharmacokinetics and immune response potential when these antibodies were dosed SC into rodents. We speculate that factors (intrinsic such as charge and pI as well as extrinsic such as buffer osmolarity and excipients) that increase protein-protein interaction will negatively impact SC absorption and immunogenic potential. However this correlation still requires extensive investigation.

6.2. Buffer conditions

Charge interaction within the SC space may be critical for PK absorption, however few studies have illustrated this. As highlighted in a detailed review by Kinnunen and Mersny and discussed previously, the chemical and physical characteristics of proteins within formulations should be critically explored as to their interaction within the injection site ¹⁴². Osmotic difference at the site of injection leads to water flushing out to maintain physiologic pressure, and small excipients may be flushed away in the process away from the larger protein. It is proposed that in some circumstances a critical excipient is lost via this process and protein could rapidly aggregate before being properly absorbed ¹⁴². The opposite might also occur, as the excipient undergoes an affinity change while within the SC injection site towards the therapeutic or some component in the ECM. For example, an unwanted affinity increase within this space of a surfactant excipient might cause an elevation of concentration beyond the critical micelle concentration. This could put the therapeutic at risk for detergent mediated denaturation, and perhaps eventual aggregation leading to rapid clearance or immunogenicity ¹⁴²

Certain formulation conditions can mask or counteract the charge of proteins within solution. This charge altering effect can be intended for storage considerations involving long term stability or aggregation. However, within the SC space the stabilizing buffer may behave differently or alter protein stability or SC absorption¹⁴², and generally this phenomenon is protein specific based on physical properties. One example of formulation buffer altering charge illustrates that citrate ions containing two negative charges in solution with a positively charged protein can reverse its overall charge to negative through electrostatic interactions¹²⁶. For negatively charged proteins, buffers of high ionic strength may neutralize surface charge due to high concentrations of positive ions such as sodium seen in citrate or phosphate buffers. Within the same study, Mach and colleagues determined that higher ionic strength of oppositely charged ions, rather than simple electrostatic interaction, increased the percent of free protein within a solution of rat SC tissue. This effect is compounded further by changes in pH, as increasing pH caused a subsequent reduction in SC space tissue binding.

Certain ions can destabilize a protein to the point of unfolding, representing denaturation and possible aggregation and deactivation. Carbonate has been reported to be one buffer able to stabilize protein structure, unless the charge of the protein is positive^{143,144}. Being that carbonate buffers are closer to pH 9, it may be difficult or impossible to stabilize proteins such as mAbs in this manner. A buffer screening method developed by Casaz and colleagues has revealed that acetate and succinate buffers provided better stability for mAbs than citrate, histidine, or phosphate buffers¹⁴⁵. In an unpublished study from our research team, slight differences in melting temperature and aggregation potential were seen when only the buffer species (citrate vs histidine vs acetate) within a formulation was altered, with histidine buffer resulting in reduced protein-protein interaction and improved stability. Further, dosing a model mAb using histidine resulted in a significantly higher AUC compared to citrate or acetate, however we would emphasize that this is likely a molecule specific event (unpublished). Buffers not only play a role in storage stability, but may also influence absorption and immunogenic properties for a variety of protein therapeutics.

Surfactants such as polysorbate-20 and 80 are added to reduce protein-protein interaction by binding to hydrophobic regions and preventing surface degradation, a more recent adaptation opposed to previous use of human serum albumin which carried with it associated immunogenicity¹⁴⁶. In some formulations sugars such as polyol osmolytes are used as stabilizers, generally resulting in increased melting temperatures during biophysical testing^{147,148}. In addition, salt solutions such as NaCl are added to adjust osmolarity, increase surface tension of the formulation, and maintain chemical potential¹⁴⁹. Taken together, formulation co-solvents offer stability through various molecular interactions and mechanisms.

6.3. Formulation excipients and additives

Some excipient interactions within the ECM may be considered beneficial for PK, with the possibility of increasing SC absorption by interacting with the ECM in a way that prevents nonspecific binding by the administered protein. Hyaluronidase is one such additive that catalyzes the hydrolysis of the hyaluronic acid component of the ECM while being safe and

completely reversible^{150,151}. This lowers the viscosity of hyaluronan and increases tissue permeability by destabilizing the fibrinogous network. It has been used therapeutically in surgery and ophthalmology as an anesthetic enhancer and intraocular pressure countermeasure respectively, however new applications have sprung up for enhancing delivery of protein therapeutics^{150,152}. Recombinant human hyaluronidase has been used in conjunction with rapid-acting insulin to accelerate SC absorption and reduce the intrasubject variability of said absorption¹⁵³. In an overview of recombinant human hyaluronidase by Wasserman, compared with IgG administered alone, a 20.4% improvement to bioavailability was seen with the addition of the enzyme with a delayed absorption rate¹⁵⁴. Another study of IgG serum levels after intradermal administration showed a large increase in absorption rate and bioavailability with the addition of hyaluronidase¹⁵¹. Rituximab SC 1400 mg (MabTheraR) formulated with hyaluronidase replicates IV infusion trough level plasma concentrations and can be administered at volumes up to 11.7 mL over 2–8 minutes safely and effectively¹⁵⁵. Trastuzumab 8 mg/kg formulated with hyaluronidase resulted in exposure profiles mimicking the approved 6 mg/kg IV dose, while allowing injection volumes nearing 5 mL¹⁵⁶. The clinical success of these products may evolve into a new formulation standard for future SC therapeutics. Most recently, a hydrogel system consisting hyaluronidase and trastuzumab has been formulated that takes advantage of not only the benefits of hyaluronidase but also controlled release dosing as a long term infusion¹⁵². Unfortunately, case studies have shown a risk of immunogenicity against hyaluronidase. A clinical trial testing immunogenicity potential found that although incidence of anti-hyaluronidase antibodies were upwards of 18%, no neutralization or unwanted side effects were found in association¹⁵⁷.

Several studies have observed an increase in lymphatic uptake by altering the osmotic pressure of an injected volume containing a biotherapeutic, indicating that formulation buffers consisting of hypertonic solution may be beneficial in increasing rate or extent of absorption, perhaps effecting the bioavailability of SC administered proteins. In one example Albumin was used as a “volume expander” to increase lymphatic uptake of recombinant human interferon- α 2¹⁵⁸. Altering the oncotic pressure in the ECM can further prevent fluid reabsorption at the post capillary beds, increasing the interstitial volume and driving lymphatic uptake before being diluted within the injection space¹⁵⁹. In another study from our research group, hypertonic buffers at various osmotic strengths of OPLS and mannitol revealed that lymphatic uptake of rituximab was improved for SC administration, which in turn increased absorption and relative bioavailability by 28 or up to 70%⁹⁵. Tonicity agents such as NaCl are thought to have minimal impact due to rapid distribution immediately after injection, however for non-permeable molecules such as OPLS and mannitol depend on transporters for distribution and therefore remain at the site of injection longer. This leads to a more prolonged state of hyperosmolarity which forces hydrostatic pressure rebalance by increased fluid uptake into the ECM, which then is alleviated by lymph drainage and subsequent protein uptake⁹⁵. The impact of osmolarity on SC absorption and bioavailability of mAbs in rodent models is likely to be molecule specific based on biophysical characteristics and highly variable, as this effect was not reproduced for another model mAb in a separate unpublished study. A unified conclusion could not be drawn based on rodent studies, partially due to differences buried in variability that includes buffer species used,

massaging an administered dose immediately after injection, and analytical differences in concentration analysis. Inter animal variability can further skew conclusions, which complicates overall conclusions.

7. IMPROVING THERAPEUTIC OUTCOMES OF SC ADMINISTERED PROTEINS: SUMMARY AND CONCLUSIONS

As interest in protein therapeutics continues to grow with new treatment approaches on the horizon, there exists a need for better prediction tools and interdisciplinary focus among industry, regulatory agencies, and academic institutions. As therapeutic options move toward SC drug formulations to accommodate patients, it is of utmost importance to consider all of these major characteristics (formulation, immunogenicity, and pharmacokinetics) when designing and optimizing new SC protein formulations. Although each respective category holds merit, development teams to be all-encompassing, or risk losing valuable resources at later clinical phases. Many examples of interactions between formulation considerations and their impact on immunogenicity and/or pharmacokinetics have been discussed here. Favorable PK and immunogenicity profiles are highly variable between proteins and formulations, requiring an understanding of injection site interactions, formulation excipients, degradation potential, and drug specific parameters. Overall, these challenges are interrelated and require an all-enveloping methodology to better predict respective outcomes in terms of efficacy and safety. A holistic approach has been emphasized as the best way to overcome the challenges facing SC delivery, and would improve biopharmaceutical development if acknowledged.

One of the biggest hurdles to the development of SC therapeutic proteins is human *in vivo* prediction from early development stages. Whether this is estimating effective absorption and bioavailability or determining potential immune responses, current approaches have not always lead to desirable outcomes. One of the biggest challenges is the lack of a standard, universal *in vitro* system that accurately represents all facets of the SC space. While several *in vitro* and *ex vivo* skin models have been described here, lack of cohesion with all relevant systems is a recurring flaw. In addition, caution must be applied when using animal models for predictive purposes and scale-up approaches, as biotherapeutic absorption varies wildly species to species. While a full scale model that incorporates immune response, skin homology, fluid dynamics and extracellular makeup may not be possible due to complexity, it is important that these features are all considered during development and testing, lest a critical factor be overlooked. New therapeutics should be designed from multiple angles, resulting in products with low immunogenicity and appropriate pharmacokinetics, within formulations designed to increase stability and reduce aggregation both during storage and upon injection.

Acknowledgments

The authors gratefully acknowledge financial support from the National Institutes of Health (R01 HL- 70227). We would like to thank the following people for carefully reviewing the article and providing helpful comments and suggestions: Fiona Yau and Robert Dingman. The opinions expressed in this review are those of the authors and do not represent the opinion or position of The University at Buffalo, FDA, NIH, or any other corporate entity.

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Figure 1.

Figure 2.

Table 1
Approved Protein Therapeutics with IV and SC routes: Differences in Concentration, Bioavailability, Immunogenicity Rate, and Formulation Composition

Product	Description/Target	Protein Concentration (IV vs SC)	Bioavailability (%)	Immunogenicity Rate (%)/ADA development	Formulation
Adalimumab ¹⁶⁰	Human IgG1/TNF α	40 mg/infusion vs 50 mg/mL	64 %	SC 1–12 %/NA	Sodium chloride, monobasic sodium phosphate dehydrate, dibasic sodium phosphate dehydrate, sodium citrate, citric acid monohydrate, mannitol, 0.8 mg PS80, pH 5.2
Infliximab ¹⁶¹	Chimeric IgG1/TNF α	10 mg/mL vs 100 mg/injection	N/A	IV 10–27 %/NA SC 5–27 %/low-tier	Sucrose, monobasic sodium phosphate, dibasic sodium phosphate, PS80, pH 7.2 after reconstitution
Rituximab ¹⁶²	Chimeric IgG1/CD20	10 mg/mL vs 120 mg/mL	65 %	IV 3–4 % ADA ¹⁶³ , 10.6 % anti-chimeric antibody ¹⁶⁴ SC 2–18 % non-nADA ¹⁶⁵	Recombinant human hyaluronidase (rHuPH20), L-histidine, L-histidine hydrochloride monohydrate, α - α -trehalose dihydrate, L-methionine, PS80, pH 6.5
Trastuzumab	Humanized IgG1/HER-2	21 mg/mL vs 30 mg/mL ¹⁶⁶	77.1 %	IV 8.1 %/<1 % nADA SC 14.9 %/ 1–2% nADA	IV: α - α -trehalose dihydrate, L-histidine HCl monohydrate, L-histidine, and PS20, pH ~6.0 SC: Recombinant human hyaluronidase (rHuPH20), L-histidine, L-histidine hydrochloride monohydrate, α - α -trehalose dihydrate, L-methionine, PS20
Alemtuzumab	Humanized IgG1/CD52	30 mg/infusion vs 10 mg/mL ¹⁶⁷	N/A	IV 30–85 %/ nADA ^{168,169} SC N/A	Sodium chloride, sodium phosphate-monobasic, potassium chloride, potassium phosphate-monobasic, PS 80, disodium edetate, pH 7.0–7.4
Interferon β ¹⁷⁰	rProtein/Interferon	N/A vs. 0.25 mg/mL	27 % ¹⁷¹ 50 % (FDA)	2–47% IV depending on product nADA ¹⁷⁰ 45% SC ¹⁷² 5.3% IV vs 16.3% SC nADA ¹⁷³	Albumin (human), mannitol, sodium acetate (lyophilized).
Darbepoietin α ¹⁷⁴	Cytokine/EPO analogue	25–500 μ g/infusion vs. 60– 500 μ g/mL	30–50% in adults	4 % /non-nADA	Sodium phosphate monobasic monohydrate, sodium phosphate dibasic anhydrous, sodium chloride, PS80, pH 6.0–6.4
Factor VIIa ¹⁷⁵	rProtein/Coagulant	0.6 mg/mL vs. 15 mg/mL	20–30 % Dose mediated	11 % /non-nADA ¹⁷⁶	Sodium chloride, glycylglycine, PS80, calcium chloride, mannitol, pH 5.5

Product	Description/Target	Protein Concentration (IV vs SC)	Bioavailability (%)	Immunogenicity Rate (%) / ADA development	Formulation
Emicizumab ¹⁷⁷	Humanized bispecific antibody/Factor VIII mimetic	N/A vs. 80 mg/mL	102.3% in cyno monkeys	4.2 % / non-IgE ¹⁷⁸	N/A
Etanercept ¹⁷⁹	Fusion Protein/TNF α	N/A vs. 50 mg/mL	76 % ¹⁸⁰	6 % / non-nADA	Lyophilized powder: mannitol, sucrose and trometamol. pH 7.1–7.7 Solution for injection: sucrose, sodium chloride, L-arginine hydrochloride, sodium phosphate-monobasic dihydrate, sodium phosphate-dibasic dihydrate. pH 6.1–6.5 ¹⁸⁰
Abatacept ¹⁸¹	Fusion Protein/Prevention of T-cell activation	<10 mg/mL vs. 125 mg/mL	78.6 %	5.8 % / 67% of those nADA	Dibasic sodium phosphate, anhydrous, monobasic sodium phosphate, monohydrate, poloxamer 188, sucrose. pH 7.2–7.8
rHuman Insulin (various)	Peptide Hormone/Glycoprotein Receptor	U-100 Insulin: 0.1–1 IU/mL vs. 100 IU/mL	40–100% depending on type ¹⁸²	SC 14–44% non-nADA ^{183,184}	(Various), commonly glycerin, metacresol (zinc-oxide), phenol, disodium hydrogen phosphate dehydrate, sodium chloride. pH 7.0–7.8 ^{185,186}

ADA, anti-drug antibody; nADA, neutralizing anti-drug antibody; non-nADA, non-neutralizing anti-drug antibody; TNF, tumor necrosis factor; EPO, erythropoietin; PS, polysorbate; CD, cluster of differentiation. Bioavailability, immunogenicity rate, and ADA development reflective of references listed.