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Improving the outcomes of biopharmaceutical delivery via the subcutaneous route by understanding the chemical, physical and physiological properties of the subcutaneous injection site

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Abstract

Subcutaneous (SC) injection is currently the most common route of self-administering biopharmaceuticals such as proteins and peptides. While pharmaceutical scientists have acquired great skill in identifying formulations for these as proteins and peptides with multi-year shelf life stability, the SC injection of these formulations can result in inconsistent or particularly low bioavailability outcomes. We hypothesise that upon injection, the chemical, physical and physiological properties of the subcutaneous tissue may play a crucial role in determining the therapeutic outcomes of SC injected biopharmaceuticals. We contend that physical and chemical stresses placed upon the injected protein or peptide as is transitions from the non-physiological environment of its formulation to the homeostatic conditions of the SC tissue can affect its fate following injection. In this mini-review we describe how events that occur to an injected protein or peptide during this post-injection transition period could affect the diffusion of bioactive material to blood capillaries and lymphatic vessels. With this in mind, we have reviewed the chemical, physical and physiological attributes of the SC tissue and collated studies on how these properties are known to affect protein stability and diffusional properties. Finally, examples where the understanding of the properties of the SC tissue when formulating for SC injected biopharmaceuticals has improved the predictability of drug delivery via the SC route are discussed, with the need for novel tools for rational and informed formulation development is highlighted.

Keywords: Biopharmaceuticals, subcutaneous injection, formulation

Introduction – subcutaneous tissue and variability of delivered biopharmaceuticals

The subcutaneous (SC) injection site is positioned below the dermis where it functions in energy storage and hydration as well as a thermal insulator and shock absorber to protect underlying musculoskeletal structures [1]. SC tissue is composed of two different tissue types; loose connective tissue, also known as areolar tissue, and underneath that, adipose tissue [2]. Blood capillaries to sustain the viability and nerve fibres with various types of endings to provide the skin with its critical sensory function permeate the SC tissue [3]. A lymphatic capillary bed resides within the SC space and there is very little proteolytic activity in the subcutaneous tissue [3]. These features make SC tissue an ideal route for administering biopharmaceuticals that are not suitable for oral delivery due to proteolytic degradation within and/or limited absorption across the epithelial barrier of the gastrointestinal tract. The SC route, however, introduces other uncertainties in drug delivery, such as variable bioavailability between different formulations and various injection sites of the body [4].

Patients are not administered drugs, they are administered formulations that contain a drug and subcutaneously injected biopharmaceuticals are often formulated in non-physiological conditions that function to improve shelf-life stability [5, 6]. Indeed, biopharmaceuticals intended for SC injection are commonly formulated at acidic pH with a variety of stabilizing agents [6, 7]. One can assume that upon administration the SC injection site returns to homeostasis condition of the body, with the temporal parameters of this recovery being dependent upon injection volume and formulation composition. We hypothesize that this period of transition from the formulation environment to the homeostatic environment of the SC tissue can be detrimental for some biopharmaceuticals, in particular proteins and some peptides. Such detrimental changes to proteins and peptides injected into the SC space, due to alterations in their structural properties could adversely affect their functional properties. Further, we hypothesize that these changes could alter the ability for an injected biopharmaceutical to be absorbed efficiently from the SC injection site.

Our hypotheses related to the potential for a period of possible instability during the transition from environmental conditions of an injected formulation to the homeostatic condition of the body provides a potential explanation for the observation that some proteins and peptides injected into the SC space have bioavailability outcomes that are unacceptably low or variable. In that light, the current review article aims to summarise the chemical, physical and physiological characteristics of the subcutaneous tissue, highlighting the need for considering not only shelf-life stability but also the properties of the subcutaneous tissue in the optimization of protein and peptide formulations intended for SC injection. A second aspect of the review aims to illustrate how characteristics of the SC injection site may affect pharmacokinetic and/or pharmacodynamic profiles of biopharmaceuticals following SC injection. Finally, the future perspectives of SC drug delivery, formulation improvements and emerging analytical techniques for enhancing the efficacy of SC drug delivery are highlighted in the last part of the review article. It is important to note that this mini-review focuses on events occurring shortly after the injection; longer term adverse effects, such as immunogenicity that may or may not be a result of the potential post-injection stability issues [8] is outside the remit of the current review.

Physiological, physical and chemical properties of the subcutaneous injection site

The major physiological and chemical features of the subcutaneous tissue are schematically presented in Figure 1. These are the extracellular matrix formed by collagen, hyaluronic acid and

chondroitin sulfate, the interstitial fluid, the temperature of the tissue and hydrostatic and osmotic pressure. From this diagram, it can be appreciated that the SC injection site contains an organization of collagen proteins that provides a lattice network to support integrated elements of polysaccharides. These are discussed in detail in the following section.

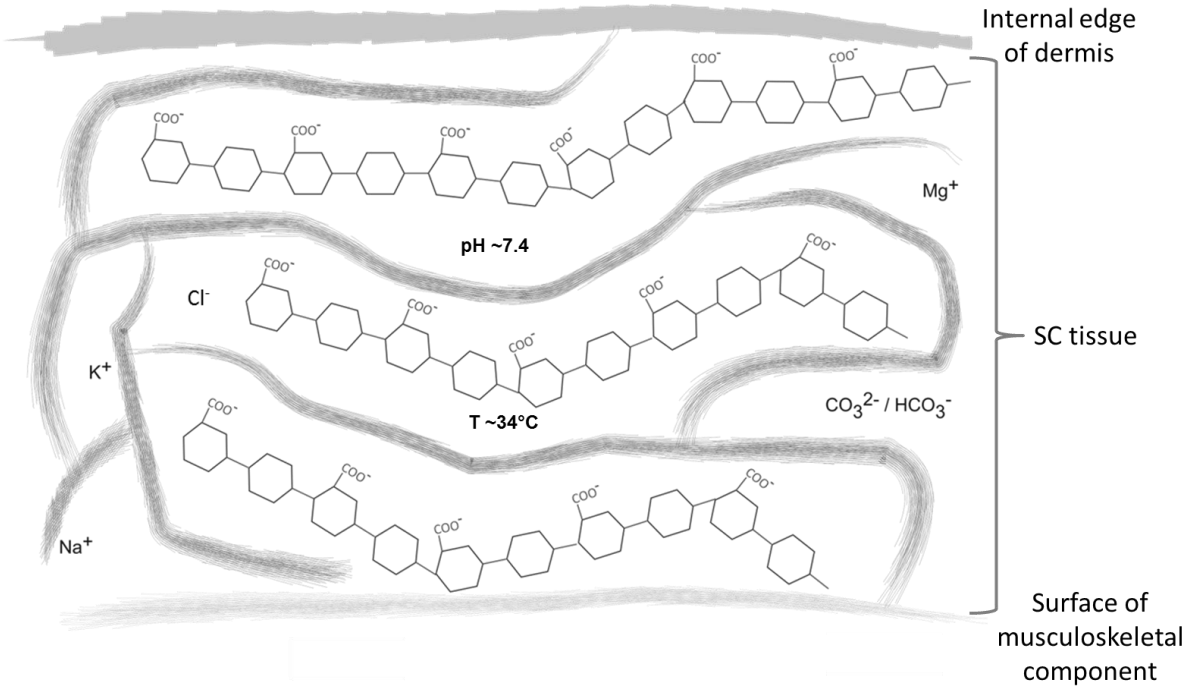

Figure 1. Diagram describing the organization of non-viable elements within the subcutaneous injection site. Stands represent network of collagen fibrils. Hexagons represent glycosaminoglycan components such as hyaluronic acid (HA) and chondroitin sulphate. For simplicity, only HA is represented with its single carboxylic acid group. Physiologically relevant ions as well as the pH and approximate temperature of this tissue are noted.

Extracellular matrix

When the extracellular matrix (ECM) was first discovered, it was described as “an amorphous ground substance” [9]. However, as the understanding of the functional properties of the ECM organisation improved it became obvious that it is a highly ordered structure with collagen providing mechanical stability in form of a three dimensional network, and glycosaminoglycans, most commonly hyaluronic acid and chondroitin sulphate, filling the void spaces within the collagen network [10]. Specific elements of the ECM will be discussed in detail in the following sections of the paper.

Collagen

Collagen is the most abundant protein within the body of mammals and forms fibrous structures that function as structural elements in the ECM, tendons and basement membranes [11]. There are several types of collagen undertaking different functions in the body that can be grouped according

to the function they have in the body: fibril forming collagens (I, II, III, V and XI), fibril associated collagens (IX, XII and XIV), microfibrillar collagen (VI), short chain collagens (X and VIII) and basement membrane collagen (IV) [12]. The most prevalent collagens in loose connective tissue, such as that present in the SC injection site, are types I and III [13].

A generalized collagen structure is illustrated in Figure 2. The common primary amino acid sequence for all collagen types is a repeating (glycine-X-Y) motif, where X and Y can be any amino acid but are frequently proline (Pro) or hydroxyproline (Hyp) residues [14]. The collagen polypeptide forms a left-handed helix with the glycine (Gly) residues positioned on the same axis throughout the helix. The imino acid residues of Pro and Hyp sterically stabilise the secondary structure known as α-chain, and three α-chains form a triple helical, closely packed, supercoiled collagen fibril structure with the Gly residues packed inside the triple helix [15], as illustrated in Figure 2. The Hyp residues also participate in stabilising the collagen fibril structure in aqueous solution by enabling water bridge formation between its hydroxyl groups located in adjacent α-chains [14, 16]. The diameter of a typical collagen fibril is approximately 1.4 nm and length approximately 300 nm [17].

Collagen fibrils in the ECM are associated in bundles via interactions mediated by proteoglycans, forming collagen fibres that are between 1 and 100 μm thick. The fibrils taking part in a bundle can branch and the branched bundle in turn can take part in fibre formation elsewhere, thus creating a branched collagen network [18]. When observed with microscopic techniques, D-periodicity that is a structure where neighbouring fibres are displaced from each other axially with a distance of D due to electrostatic and hydrophobic interactions between the adjacent fibrils, is characteristic of collagen [12], as illustrated in Figure 2. The isoelectric point of type I collagen has been reported as approximately 10 [19], meaning that at physiological pH, collagen carries a net positive charge; the net charge of collagen at physiological pH and ionic strength has been measured as +14 mV [20]. Collagen concentration in the subcutaneous tissue has been reported to be 55% of dry weight in rat subcutaneous tissue [21], and 50% of dry weight in normal human skin [22]. To the authors' best knowledge, the collagen concentration in the subcutaneous tissue of man has not been reported in the literature.

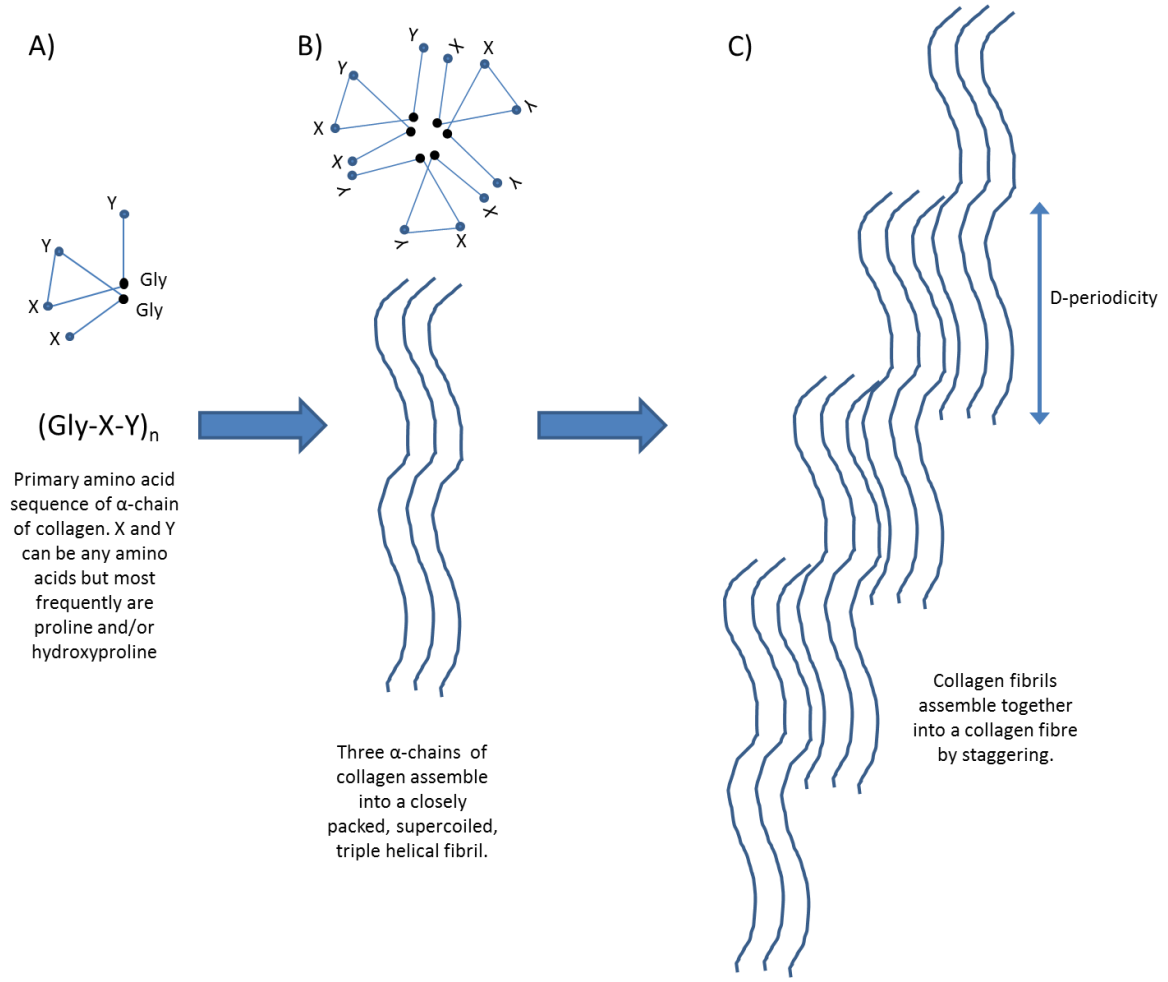

Figure 2. A schematic of collagen fibre assemblies in subcutaneous tissue. A) Organization of amino acids in the repeating collagen α-chain sequence and left handed helical structure assembly with glycine (Gly) residues stacking on the same axis and X and Y (most commonly proline and hydroxyproline) forming the outside edge of the chain. B) Organization of three α-chains assembled into a collagen fibril with Gly residues of adjacent chains packing into the center of the helix fiber to expose and X and Y residues at the outer surface. C) Assembly of fibrils into a D-periodic collagen fibre.

Hyaluronic acid

Hyaluronic acid (HA), also known as hyaluronan or hyaluronate, is a mucopolysaccharide that fills in the void spaces within the collagen fibre network, as illustrated in Figure 1. The chemical structure of HA is shown in Figure 3. The basic building block of this biopolymer is a disaccharide unit formed of N-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA) linked together via a β1-4 glycosidic bond. These disaccharides are, in turn, joined by β1-3 bonds to form a linear (unbranched) polysaccharide that can have a molecular weight of up to 2000 kilodaltons (kDa) [23-25]. HA is also capable of forming ordered tertiary structures, such as double helixes [26].

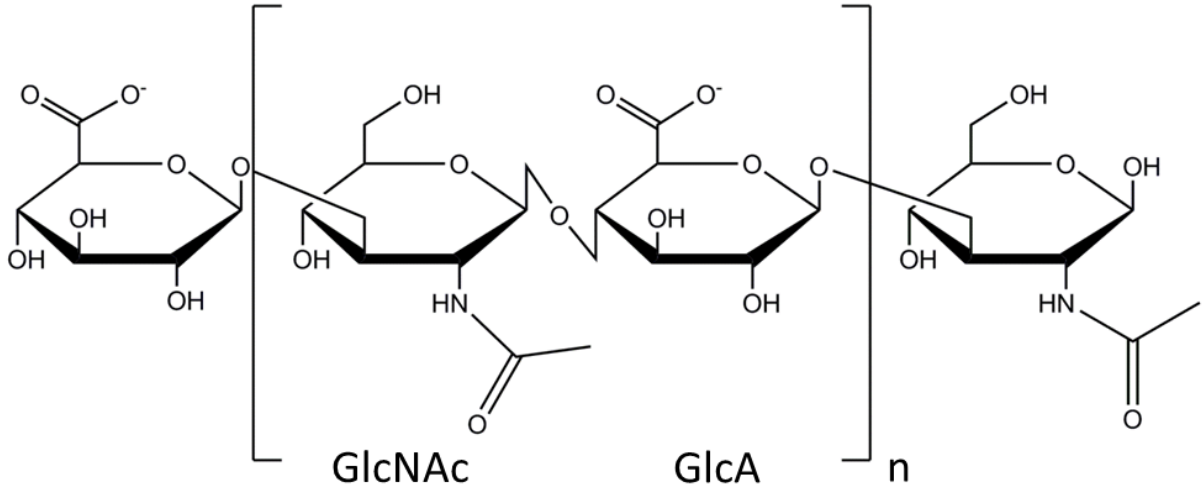

Figure 3. Chemical structure of hyaluronic acid. The repeating disaccharide unit is composed of N-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA) that are linked by a β1-4 glycosidic bond. These disaccharides are joined together by β1-3 bonds to form a linear, unbranched polysaccharide. At physiological pH, the carbonate groups of GlcA carry a negative charge.

In addition to the large molecular weight of the molecule, another remarkable feature of HA is that at physiological pH and ionic strength, the carboxylic acid groups of GlcA molecules predominantly carry a negative charge [23]. The polyanionic nature and large molecular weight of the molecule contribute to the function of HA within the body in providing matrix structures, maintaining water balance of the body, regulating the distribution of proteins in the tissues by steric exclusion and, due to its unique rheological properties, lubricating joints and tissues [24].

The concentration of hyaluronic acid in the subcutaneous tissue is approximately 1 gram in 100 grams of wet tissue [27]. HA is degraded within the body and the relatively rapid half-life of HA in the tissues varies between half a day and three days. Degraded HA components are taken up by the lymphatic system [25].

Chondroitin sulfate

The chemical structure of chondroitin sulfate (CS), a linear glycosaminoglycan polysaccharide that is composed of repeating disaccharide units of D-glucuronic acid (GlcA) and D-galactosamine (GalN), is shown in Figure 4. The D-galactosamine units are sulfated either at C-4 or C-6 positions of the polysaccharide chain producing either chondroitin-4-sulfate or chondroitin-6-sulfate, respectively. The molecular weight of CS in tissues is often between 30 and 40 kDa [28]. Similar to HA, CS is also negatively charged at a physiological pH. However, the charge density of CS is twice as high as that of HA due to the fact that both GlcA and GalN residues are anionic in a CS molecule while HA carries a single negative charge per disaccharide unit [29].

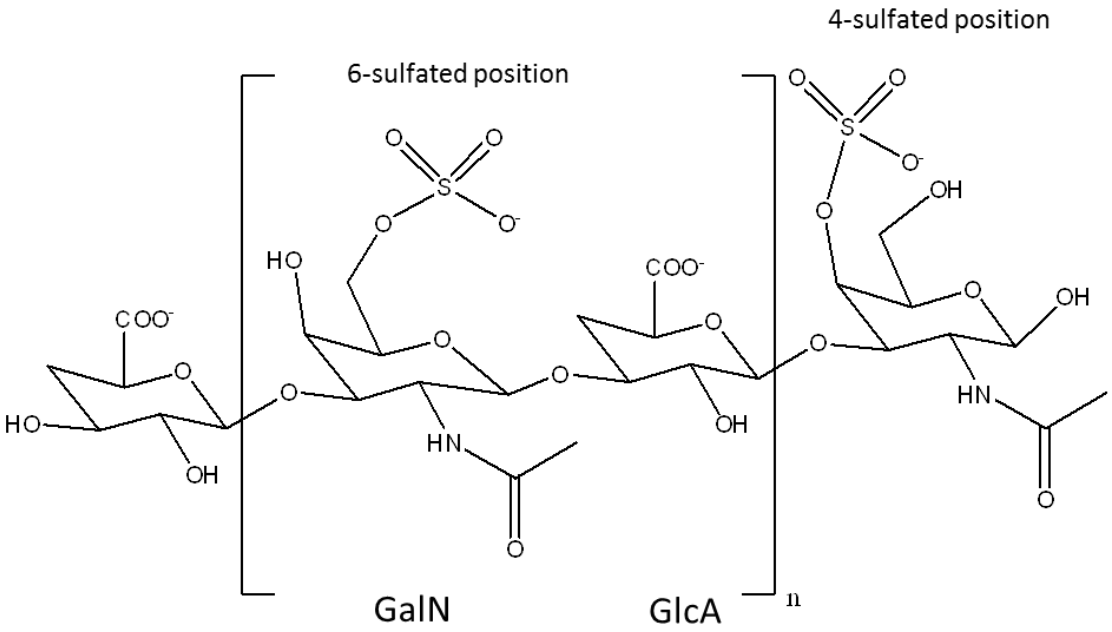

Figure 4. Chemical structure of chondroitin sulfate (CS). The repeating disaccharide unit of CS is composed of D-glucuronic acid (GlcA) and D-galactosamine (GalN). Possible sulfation positions in the D-galactosamine unit (C-4 or C-6) are illustrated.

It has been suggested that CS may act as a viscosity modifier within the ECM, as it has been shown that the viscosity of chondroitin sulfate solutions on their own is very low, while the addition of CS into HA solution increases the viscosity of the HA [30]. It has also been suggested that CS participates in water uptake and nutrient absorption within the tissues [28]. There is a paucity of information on the CS concentration in subcutaneous tissue. However, SC tissue is classified as loose connective tissue, and the ratio of HA to CS in Wharton's Jelly, another tissue classified as loose connective tissue, has been reported as approximately 2 in 1 [31].

Interstitial fluid

The subcutaneous space is perfused with the interstitial fluid, the body fluid that is not held within cells or in blood plasma, and that accounts for approximately 25% of total body water in man [32]. The pH of interstitial fluid is maintained at approximately pH 7.4 [33]. Factors that contribute to the pH regulation of the interstitial fluid are the dissociation constant of water, the strong ion difference ([SID]) and the partial pressure of carbon dioxide in the tissue, while also fulfilling the principle of electrostatic neutrality [34]. As illustrated by Table I, the main cation dissolved in the interstitial fluid is sodium with minor amounts of potassium, magnesium and calcium also present. The main non-volatile anion is chloride with a minor proportion of sulphate. Notably, phosphate has been omitted from the table as its concentration in extracellular body fluids is negligible [34].

Table I. The concentrations of different anions and cations in the interstitial fluid. Adapted from [34].

Cation	Concentration (Eq/l)	Anion	Concentration (Eq/l)
Na^+	0.137	Cl^-	0.111
K^+	0.003	HCO_3^-	0.031
Mg^{2+}	0.002	SO_4^{2-}	0.001
Ca^{2+}	0.001	CO_3^{2-}	0.000045
Total cations	0.143	Total anions	0.143

The concentration of sodium ions is higher than the concentration of chloride ions, thus resulting to a positive strong ion difference [SID] of the interstitial fluid. As a result, without dissolved carbon dioxide (CO_2), the pH of the extracellular fluid would be approximately 11.9 [33]. However, at positive [SID], the presence of gaseous carbon dioxide, which is a product of cell metabolism, decreases the pH of the solution due to the favoured formation of bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions to neutralise the positive [SID]. Consequentially, the interstitial fluid pH in a closed system is only regulated by the [SID] and the partial pressure of CO_2; addition of H^+ ions into this closed system does not significantly change the pH [34].

Within the SC tissue, the interstitial fluid is not a closed system but is in an equilibrium with the intracellular fluid and blood plasma compartments [34]. Following cell metabolism, CO_2 and weak acids, such as lactate, will be excreted from the cells into the interstitial fluid. The increase in CO_2 does not alter the pH of the interstitial fluid as CO_2 is rapidly diffused into the plasma and dealt with by the respiratory system. However, the increase in weak acids, such as lactate, will increase the [SID] and consequentially decrease the pH. The decrease in pH is counteracted by exchanging other strong ions between the cellular and interstitial compartments to neutralise the [SID] and thus a physiological pH is restored [34]. In the balance of all of these interacting factors, the interstitial fluid bicarbonate concentration is maintained at approximately 25 mM [35].

Pressure

SC tissue interstitial pressure is primarily regulated by two factors: the interstitial fluid hydrostatic pressure P_{IF} caused by the limited volume of the sc space and the colloid osmotic pressure P_{CO} exerted by plasma proteins through the semipermeable capillary wall. P_{IF} and P_{CO}, in conjunction with lymphatic flow, control the volume of the interstitial fluid, and thus the pressure, in the tissues [36, 37]. SC tissue values of P_{IF} have been reported as slightly negative at –1 to -2 mmHg [37, 38] and the elastic nature of the dermis allows for changes in water content of the SC space to occur while maintaining the P_{IF} within a normal physiological range [39]. Positive values of P_{IF}, however, are associated with different pathological conditions, such as oedema [38], as such events result in fluid accumulation that exceeds the elastic properties of the overlying dermis and underlying musculoskeletal structures. Thus, changes in the P_{IF} value are normally connected to changes in the fluid volume within the SC tissue; increased interstitial fluid volume can result in an increase of P_{IF} [36].

P_{CO} is, in essence, the difference between colloid osmotic pressures of plasma and interstitial fluid [37]. The colloid osmotic component (P_{CO}) has been reported as 15.8 mmHg in the subcutaneous tissue of thorax [40] and 10.2 mmHg in the SC tissue of the ankle [41] in man. P_{CO} may be altered in

some pathological conditions where plasma protein leakage through the capillaries occurs, i.e. pre-eclampsia [42].

Temperature

Temperature of the human body is generally regarded as approximately 37°C. However, this is the temperature of the body's core and temperature of the SC tissue at comfortable environmental temperature (~20°C) have been shown to average approximately 34°C [43, 44]. Sites closer to the body's core, such as chest and back, demonstrate slightly higher temperatures than the extremities such as thighs, calves, feet and arms [44]. Importantly, one of the roles of the SC tissue is systemic thermal regulation. Consequentially, SC tissue temperature has been shown to average 30°C on the onset of shivering and 36°C on the onset of sweating [44]. In this way, SC tissue temperatures are typically maintained within a range of 30-36°C.

Potential stressors experienced by a biopharmaceutical upon transition from the formulation environment to the environment of the SC injection site

Extracellular matrix binding interactions

To be absorbed into the systemic circulation in a biologically active form, a biopharmaceutical must remain sufficiently stable following its SC injection prior to uptake into blood capillaries and lymphatic vessels. Depending on the glycosaminoglycan concentration of SC tissue and molecular weight of the solute, molecules within the SC tissue can be transported either by diffusional or by convective flow [27]. ECM components of the SC injection site, however, can pose a resistance to the solute flow. As highlighted in the previous section, the ECM is composed of a collagen network with void spaces filled with HA and CS. These components determine the tissue's resistance to flow via a complex interplay; it has been shown in experimental studies with model ECMs that the resistance of SC tissue to flow is lower than that of a model collagen network or of a model HA matrix at physiologically relevant concentrations [45]. Combining the effect of HA and collagen was still found to underestimate the flow resistance of the tissue, and theoretical models have shown that the presence of proteoglycans, such as CS, add significant flow resistance in the tissues [45].

Steric exclusion

Steric exclusion of a biopharmaceutical entity may be one of the mechanisms influencing the rate of drug and excipient transport, and thus absorption, from the SC injection site. For example, hyaluronic acid has been shown to act as a molecular sieve and thus affect diffusion rates of proteins through HA solutions, with both increasing concentration of HA and increasing size of a globular molecule transported through the matrix resulting in a decreased diffusion rate [46]. Steric exclusion has also been attributed to account for the differences observed in diffusion rates of macromolecules of different shapes through HA with elongated particles shown to diffuse faster than globular particles of similar molecular weight [47].

Steric exclusion by HA may also affect protein solubility at the SC injection site. It has been shown that when added in a solution of HA, steric exclusion of proteins takes place and consequentially, the solubility of proteins is decreased with larger proteins being more affected than smaller ones [47,

48]. This effect is dependent on the HA concentration with higher concentrations producing lower protein solubilities [49]. Therefore, it may be that, due to their larger size (up to 150 kDa) and generally high concentration formulations (up to 150mg/ml) [6], monoclonal antibodies (mAbs) injected into SC tissue may be especially susceptible to precipitation via the mechanism of steric exclusion.

Electrostatic interactions

Electrostatic interactions have also been suggested to play a role in regulating macromolecular diffusion through HA matrices. Diffusional rates of different macromolecules in HA and HA-collagen matrixes are dependent on the HA concentration with increasing HA concentration posing more hindrance on diffusion both in the presence and absence of collagen [50]. In the presence of collagen, however, the extent of hindrance to diffusion posed by the HA was found to be reduced compared to aqueous solutions of HA at equivalent concentrations. This was explained by neutralisation of some of the negative charges of HA due to interactions with the positive charges on collagen [50].

Electrostatic interactions may also influence the stability of injected biopharmaceutical. Indeed, a theoretical study indicated that proteins form electrostatically stabilised complexes with polyelectrolytes and that the formation of these complexes is mainly regulated by the surface charge of the proteins [51]. Experimental work proved that complex formation between a polyelectrolyte and a protein may take place even close to the isoelectric point (pI) of the protein where the net charge is essentially zero but regional charged patches would still exist [52, 53]. In a separate study it was found that positively charged therapeutic antibodies bound to homogenised rat SC tissue in a way that was dependent of both pH and the ionic strength, indicating that electrostatic interactions were playing a role in determining the extent of absorption of these antibodies from the SC injection site [54]. The relationship between the pharmacokinetic (PK) and pharmacodynamic (PD) properties of intravenously administered mAb formulations and protein charge have also been investigated, and it was concluded that after extravasation, electrostatic ECM-protein interactions played a part in determining PK/PD properties [55, 56].

It is clear that the transport of molecules though the ECM is a complex phenomenon and regulated by a variety of mechanisms, such as steric exclusion and electrostatic interactions. A computational study indicated that there is a complex interplay between the interactions and the diffusing entities; for particles that were significantly smaller than the ECM pore structure, electrostatic effects appeared to be the most significant determinant of diffusion rates as neutral particles were seen to diffuse more rapidly than cationic particles. For nanoparticles with size similar to the pore size of the ECM, steric hindrance became significant and the charge of the particles was negligible in determining the diffusion rate [57]. Consequentially, in biopharmaceutical formulations, the rate and the mechanism regulating the transport rate of the active ingredient (API) may be completely different compared to those observed for stabilising excipients present in the formulation.

Specific versus non-specific interactions

It has been suggested that one of the functions of the extracellular matrix is to store biologically active molecules, such as growth factors, so that they are readily available when adjacent cells require them [58]. Therefore, it is not surprising that one of the factors affecting uptake of a

biopharmaceutical from the SC injection site may be its binding to ECM components. For example, HA has both non-specific and specific interactions with proteins, with the specific interactions being ascribed to certain HA functions within the body and the non-specific interactions due to the polyelectrolyte nature of HA [59]. Specific HA binding partners are most commonly found in cartilage where HA plays a role in stabilising the structure [60], or on cell surfaces where interactions between cells and the ECM are mediated via receptors such as CD44 [61].

Proteins involved in these interactions, called hyaladherins, bind HA; hyaladherins contain a linking module that is a structural domain composed approximately of 100 amino acids enriched in basic amino acids, such as arginine, which may bind HA via electrostatic interactions [62]. However, some proteins lacking this linking module still bind HA [62], and several studies have shown that proteins lacking specific HA binding domains, such as bovine serum albumin (BSA) [63, 64] and lysozyme [65] can form complexes with HA. In case of BSA it was shown that at physiological ionic strengths, insoluble neutral complexes and charged, soluble complexes between BSA and HA could form that were dependent upon stoichiometry; at excess HA concentrations, soluble, negatively charged, unfolded ribbon complexes of large size are formed. If the stoichiometry of HA and BSA resulted in neutral complexes, these tended to aggregate to larger precipitates due to the lack of electrostatic repulsion. At excess BSA concentrations, positively charged, soluble compact complexes were formed [64].

These studies clearly demonstrate that there is a potential of some biopharmaceuticals forming non-specific complexes with HA that may be relevant to their successful SC administration. However, in addition to binding HA, biopharmaceutical binding to collagen and CS may also take place, thus altering the therapeutic outcomes after SC injection. For example, a study by Yaoi, Hashimoto, Takahara and Kato [66] reported that insulin had a high binding affinity to collagen, especially to type V, although it was also found to bind to other collagens (e.g. types I, II and IV). Interleukin-8 (IL-8), a naturally occurring chemokine administered via SC injection to reduce inflammation, has been shown to bind to CS in the lungs via an interaction mediated by its glycosaminoglycan binding domain consisting mainly of basic amino acids. This binding was suggested to act as a mediator of the local concentration of IL-8 and the subsequent duration of action for IL-8 in the lungs in regulating inflammation [67].

Excipient-API interactions

A variety of excipients, including sugars, salts, and surfactants are used for each biopharmaceutical to achieve a multi-year shelf life required for commercial success (Figure 5A). It is conceivable that API-excipient interactions may also factor into determining the PK and PD outcomes following the SC injection of a biopharmaceutical formulation. These effects would be due to potential alterations in API-excipient interactions following the injection of a biopharmaceutical formulation into SC tissue. The primary basis for such potential effects are related to pH-dependent events associated with the transition from acidic (4-6) pH conditions frequently used to stabilize a biopharmaceutical to the neutral pH of the body following SC injection. Several potential scenarios for pH-dependent changes in API-excipient have been illustrated in Figure 5. The nature of these API-excipient changes could be due to charge and/or conformation differences of the biopharmaceutical induced by a transition from acidic to neutral pH. Importantly, this pH change can result in a swing through the pI for many protein and peptide therapeutics; a situation where proteins can be poorly soluble and prone to

detrimental aggregation [68]. The loss of the protective actions of some of the excipients could be problematic and in the other hand, their local concentration, such as that of a surfactant, could have damaging actions on the biopharmaceutical.

One potential scenario involving API-excipient interactions that could positively affect the fate of a biopharmaceutical following its SC injection involves retention of these interactions during the transition through potentially stressful environmental changes (Figure 5B). It is easy to envisage that the low molecular weight excipients would rapidly leave the SC injection site, potentially leaving the high molecular weight API at risk of deleterious associations with itself (aggregation) or with ECM components (Figure 5C). One could envision pH-dependent changes in chemical and/or physical properties of the API that might alter their affinity for some of the formulation excipients that might result in positive or negative outcomes (Figure 5D). Finally, It is possible that environmental changes occurring during the transition from the injected formation to the homeostatic conditions of the SC tissue could modify the properties of some excipient, potentially producing an environment where the API might be more or less stable (Figure 5E). Thus, interactions between an API and its excipients, between individual API molecules, or an API and ECM elements could be occurring that might affect the nature of the API at the SC injection site. Indeed, pH-dependent changes in physical properties of the API and/or the stabilizing excipient would be a likely cause for these potential changes in associations.

Excipient-ECM interactions

In an ideal situation, the interactions prevailing in the formulation are maintained within the SC tissue environment and the protein remains stable and available to be transported for absorption. In a non-optimal situation, the affinity of the excipients to the protein within the environment of the SC tissue may be diminished. While many of the mechanisms used by excipients to stabilize a biopharmaceutical in a vial can be considered generic in nature, events occurring at the SC injection site, however, could be unique for different biopharmaceuticals. For example, some excipients act as molecular crowders in the bulk solution to prevent protein aggregation in a formulation (Figure 5A; diamonds). Typically, after injection, water is removed from the SC injection site to maintain stable P_{IF} and P_{CO}. This leads to an increase in the local concentration of the biotherapeutic entity and the formulation excipients (Figure 5B). Typically, small molecular weight excipients would leave the SC injection site more rapidly than the API. If the API is not affected by this or the residence time of the critical excipient at the injection site is sufficiently long, the API could be protected during the period of environmental stress (Figure 5C). If there is an insufficient level of the critical excipient during the period of environmental stress, the injected API could have extensive interactions with ECM components or itself to result in deleterious aggregation events (Figure 5D). For example, if the critical excipient is a surfactant that functions to minimize protein-protein interactions to block aggregation, rapid loss of this material from the SC injection site could lead to a similar detrimental aggregation outcome.

It is possible that some physical and/or chemical changes in the API during the period of transition from the stable formulation to SC tissue conditions alter the API-excipient interactions (Figure 5E). One could envisage how such changes could have either positive or negative outcomes for an API. Alternately, environmental changes could cause an excipient to display a higher affinity for ECM components than to the injected protein therapeutic, causing local high concentration of an

excipient and reducing the API-excipient interaction (Figure 5F). In the case where there is an increased avidity for the surfactant after the formulation is injected SC, local concentration of the surfactant could elevate above the critical micelle concentration, put the API at risk of detergent-mediated denaturation. As another example, collagen molecules do not interact with sucrose but do bind divalent Ca^{2+} ions [20]. Chondroitin sulfate is known to bind Ca^{2+} and other divalent cations [69]. Thus, the ECM could rapidly extract divalent cations from an injected formulation under the certain conditions. It is also know that albumin binds fatty acids [70]. Therefore, some classes of surfactants present in the formulation may be preferentially bound by albumin within the SC space, leaving the therapeutic protein exposed to the SC tissue environment without the stabilising effect of the excipient. However, it may also possible that at least one of the excipients will have a high affinity interaction with the ECM components, thus eliminating any unwanted ECM-API interactions, whilst the protein remains stable within the SC tissue. In this case, the protein diffusion to the capillaries and the lymphatics for systemic absorption could be enhanced.

Ionic composition

Dissolved anions and cations have the ability to alter water structure to different extent. Hofmeister was the first researcher to report the effect and consequentially, the rank order of different cations and anions in precipitating proteins out of solution is called the Hofmeister series. His original work is translated from German in reference [71]. According to the Hofmeister series, different ions have an ability to stabilise or destabilise proteins in aqueous solutions depending on the ions' capability to disturb the hydrogen bonding network of water surrounding the protein [72]. This is because the protein is surrounded by three layers of water: the solvation layer, the transition layer and the bulk layer. In essence, the solvation layer and the bulk water are competing of hydrogen bonding with the transition layer. If an added ion strengthens the hydrogen bonding network of the bulk water, the transition layer will participate in hydrogen bond formation with the bulk water. This decreases the solvent action on the protein surface and consequentially, the protein conformation is stabilised. In contrast, if the ion destabilises the hydrogen bonding network of the bulk water layer, the transition layer will participate in hydrogen bond formation with the solvation layer. This strengthens the solvent action on the protein surface, potentially leading to unfolding [72].

Not surprisingly, the physiologically most important buffer, carbonate ion, has been reported as one of the most effective anions in stabilising bulk water, and thus protein, structure [72-74]. However, protein charge will also play a role in determining the impact of ions on the protein stability, and negatively charged proteins tend to follow the Hofmeister series while positively charged proteins frequently follow an inverse Hofmeister series [72]. Therefore, for a positively charged protein the presence of carbonate ions may have an adverse impact on conformational stability. This is particularly important as the pH of the SC space is stabilized by carbonate-based buffered system. Additionally, due to the fact that proteins exhibit both positive and negative charge patches on their surfaces, the Hofmeister effect may not always be straightforward [75], making the potential impact of the pH transition stress from a formulation to neutral pH in the SC injection site unique for each biopharmaceutical.

Despite the complexities related to non-uniform surface charge of proteins, we anticipate that in some cases the Hofmeister series may be relevant for sc delivery of biopharmaceuticals due to what is essentially a buffer exchange upon injection from the formulation buffer to the SC tissue

physiological carbonate based buffer system. The protein may not be stable in the new buffer conditions, or the properties, such as the electrostatic characteristics, of the protein may change, and consequentially the transport of the protein through the ECM into the blood capillaries and the lymphatics for absorption may be altered. For example, the results of a study by Cugia *et al.* [75] illustrate the significance of the buffer composition in determining the properties of the protein. In their study, the electrostatic motility of lysozyme was measured at constant pH of 7.15 in different buffer systems. The results were shown to be altered depending on the buffering system with phosphate and citrate producing vastly different results compared to a carbonate buffer [75].

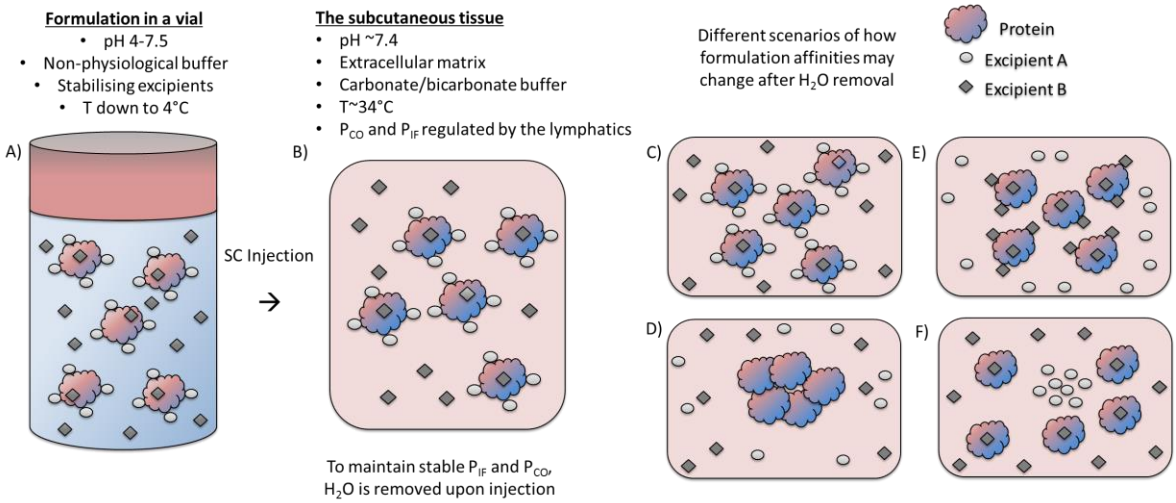

Figure 5. A schematic of potential active pharmaceutical ingredient (API)-excipient interaction outcomes following subcutaneous (SC) injection. A) Organization of components in the formulation during shelf storage depicting different degrees of API-excipient interactions. B) Upon injection into the SC tissue, to maintain stable P_{IF} and P_{CO}, water is removed from the injection site by the lymphatic system. This results to the formulation components concentrating at the injection site. Following this, multiple scenarios of protein and excipient fate are possible, as shown in the depiction of API- API-excipient interactions following removal of water from the SC injection site in a situation C) where pH transition does not strikingly affect these interactions, D) where transition to SC environment results in a rapid loss of all excipient protection with the outcome of self-aggregation and/or strong interactions with SC tissue components, E) where transition to SC tissue environment results in an exchange of excipient affinities with the API and F) where the physical and possibly functional properties of an excipient are altered following transition to SC tissue environment. The potential for different chemical and physical properties of the API between conditions in the starting formulation and the environment of the SC injection site is depicted by a transition in shading (lighter blue in the vial). For a colour version of the schematic, the reader is referred to the electronic version of the mini-review.

Tissue pH

The pH of biopharmaceutical formulations is frequently within the range from 4 to 7 [76] whereas the pH within the subcutaneous tissue is approximately 7.4 [33]. It has been reported that due to the charged nature of its components, the extracellular matrix may act as a buffer upon addition of H^+

and OH^- ions into the SC tissue [39]. Therefore, it is likely that the protein in the formulation will experience a rapid shift from the formulation pH to the physiological pH of the subcutaneous tissue. We hypothesise that in some cases, the protein stability may be compromised as a result of this shift. In fact, the pH shift from the formulation pH to the sc tissue pH has been also successfully utilised as a method for controlled release of insulin glargine by formulating the insulin with a pI of 6.7 at pH 4 [77]. Upon injection, the pH will shift through the isoelectric point of the protein causing the protein to precipitate in the presence of zinc ions. With time, the precipitated insulin slowly becomes soluble again and therefore is gradually released from the injection depot.

However, as discussed previously in this review, [SID] is the main determinant of the pH within the interstitial fluid of subcutaneous tissue. Strong ions in the interstitial fluid are Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^- , although also organic anions that are fully dissociated in the physiological pH, such as lactate, are strong ions [78]. Therefore, it may be possible that the pH of the injection site does not drastically change upon injection, unless the formulation contains strong ions as buffering agents. This is supported by a study where it was reported that the pain the patients experienced upon injection was more serious for citrate than for histidine or saline [79]. This may be because citrate is a strong ion whereas histidine and saline are not. Therefore it may be possible that the pH shift within the SC tissue upon the administration of was more significant with citrate than with saline and histidine, resulting to a more painful injection.

Tissue temperature

To improve the stability of subcutaneously administered biopharmaceuticals, the long term storage of the formulations in a refrigerator is often recommended. Patients are instructed to remove a vial from the refrigerator before usage so that the temperature of the formulation reaches the ambient temperature prior to administration. However, despite the instructions, in some cases the formulation may experience a temperature change of approximately up to 30 °C upon administration into the SC tissue. In some cases the temperature change may prove to be sufficient to cause loss of activity. One protein with such properties is recombinant human granulocyte stimulating factor (rhGCSF) that has been shown to rapidly form dimers at 37°C. However, the dimer formation could be inhibited by adding sucrose into the formulation to act as a thermal protectant [80].

Hydrostatic and oncotic pressure within the tissue

The interstitial fluid hydrostatic pressure P_{IF} is determined by two factors; the volume of interstitial fluid and the elasticity of the extracellular matrix [39]. Of these two factors, with the normal injection volumes of sc injected biotherapeutics being up to 1.5 ml for monoclonal antibodies [6], the volume of the interstitial fluid can definitely be expected to change upon injection. As a consequence, an increase in P_{IF} is expected upon injection, and it has indeed been acknowledged that SC injection volumes of 2 ml produce pressure changes that are perceived as painful [2]. Another factor regulating the P_{IF}, the elasticity of the extracellular matrix, has been reported to be governed by electrostatic interactions [39]. In some cases, upon injection of a biopharmaceutical formulation, components of the formulation may neutralise some of the charges of the ECM, and consequentially alter the elasticity of the ECM, resulting to variations in the local P_{IF} within the tissue upon injection of a formulation. Also, the colloid osmotic pressure (P_{CO}) will change as a result of a

subcutaneous injection, as the concentration of solutes impermeable to the capillaries within the subcutaneous space is increased.

As discussed earlier in the article, under normal circumstances the P_{IF} within the subcutaneous tissue is slightly negative compared to the atmospheric pressure. Under these conditions, most of the interstitial fluid is bound within the extracellular matrix. However, when positive pressures are applied, the interstitial fluid becomes more mobile. This is because to maintain stable P_{IF}, the lymphatic flow is increased [36, 81]. The changes in the P_{CO} will also stimulate the lymphatic flow to reach a new equilibrium between the plasma and interstitial compartments [82]. Mechanisms that reduce plasma volume and interstitial fluid volume by increasing the lymphatic filtration, have been reported to have maintained relatively stable P_{CO} values in patients with a variety of complications associated with diabetes [82]. Thus, SC tissue interstitial pressure is maintained within a fairly narrow range, even in the context of pathological conditions. Therefore, the SC injection of a formulation would likely result in only a transient change interstitial pressure driven by an increase in fluid volume and materials (a biopharmaceutical and excipients of a formulation) that affect the oncotic equilibrium. In the context of discussing the pressure changes within the SC tissue upon injection, it is interesting, however, that moderate pressures have been shown to affect the stability of some proteins; an effect that is pH dependent with maximal stability being observed at the pI [83]. Therefore, is some cases the pressure changes possibly taking place upon injection may have an impact on the protein stability.

Future perspectives

This review article has highlighted the importance of understanding the chemical, physical and physiological properties of the subcutaneous injection site, and how they may affect protein absorption and stability post SC administration. Understanding the significance of different interactions that may possibly take place between formulation components and SC tissue elements upon injection could enable tailoring of protein formulations to provide optimal stability not only for shelf-life but also upon SC administration. The likelihood of an injected biopharmaceutical reaching the blood capillaries and the lymphatic vessels for systemic absorption could also be improved by intelligent and informed formulations that consider diffusional barriers posed by the SC tissue; an example of a successful formulation approach that takes SC injection site chemistry into account is the addition of hyaluronidase, an enzyme that degrades the β-D-glucuronic bond in the HA molecule, into biopharmaceutical formulations. In nature, the enzyme is secreted for example as a part of snake venoms [84] and bacterial toxins [85] to aid their spread in the victim. This enzyme has been added to biopharmaceutical formulations to enable increasing injection volumes and to enhance biopharmaceutical transport through interstitial spaces as a result of the breakage of the diffusional barrier posed by HA, consequentially improving their bioavailability [86, 87]. Another successful approach in utilising the properties of the SC tissue to alter absorption and diffusion characteristics of a biopharmaceuticals is the fatty acid binding properties of albumin. In this regard, an insulin analogue has been developed to have an increased interaction with albumin, which results in decreased diffusion rate from the SC injection site to the systemic circulation and increased plasma half-life [88, 89].

One factor that has possibly limited the adoption of new formulation approaches for SC injected biotherapeutics that reflects the issues discussed in this review is that at present, there are no *in vitro* laboratory models available for predicting stability outcomes or for simulating the extent of these potential events following SC injection. Formulation testing is currently performed in animals, but these are limited in their predictive capabilities of biopharmaceutical drug stability and extent of absorption in man upon injection, possibly due to anatomical and physiological differences between species [90]. Therefore, there currently remains a need to discover and develop *in vitro* methods that would accurately model the physiological, chemical and physical properties of the subcutaneous tissue and allow predicting the stability outcomes of subcutaneously injected biopharmaceutical formulations.

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