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Insulin diffusion and self-association characterized by real-time UV imaging and Taylor dispersion analysis

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ABSTRACT

Assessment of release kinetics of subcutaneously administered protein therapeutics remains a complex challenge. *In vitro* methods capable of visualizing and characterizing drug transport properties, in the formulation as well as surrounding subcutaneous tissue environment, are desirable in drug development. Diffusion is a key process in drug release and transport. Thus, our objective was to develop a UV imaging *in vitro* method for direct visualization and characterization of insulin diffusivity and self-association behavior. Agarose hydrogels were used for mimicking subcutaneous tissue. Diffusivity, self-association, and apparent size of insulin were further characterized by Taylor dispersion analysis, size exclusion chromatography, and dynamic light scattering. At low insulin concentrations and pH 3.0, the hydrodynamic radius of insulin was determined by Taylor dispersion analysis to 1.5 ± 0.1 nm, corresponding to the size of insulin monomer. Increasing concentration and pH to 1 mM and pH 7.4, respectively, favoring insulin hexamers, increased the insulin hydrodynamic radius to 3.0 ± 0.1 nm. The UV imaging method developed was adequately sensitive to identify and characterize, in terms of diffusion coefficients, the changes in insulin transport in hydrogel due to pH and concentration changes. In conclusion, UV imaging allowed insulin diffusion in hydrogel matrixes to be studied in real-time, and showed that insulin self-association properties were reflected in the diffusion behavior. UV imaging is a useful tool for characterization of the influence of environmental conditions on protein mass transport. Hydrogels combined with UV imaging may be of utility for *in vitro* testing of protein therapeutics.

1. Introduction

Subcutaneous injection is the most common route of administration for peptide and protein therapeutics [1,2]. For the efficient development of new peptide and protein modified release formulations intended for subcutaneous administration, a thorough understanding of the drug transport in the formulation as well as in the surrounding subcutaneous tissue environment is required. Often diffusion plays a significant role in relation to drug release and transport. *In vitro* release testing methods capable of emulating as well as characterizing and visualizing these transport processes are in demand in the design and development of sustained release delivery systems. At present, standard compendial *in vitro* release testing methods do not exist for sustained release parenteral dosage forms [3,4]. A common strategy in the development of *in vitro* drug release tests for parenteral dosage forms appears to be the use of compendial apparatuses developed for oral dosage forms [5], although alternative methods have been investigated as reviewed elsewhere [5–7]. The majority of these approaches rely on the release of the active ingredient into bulk solution followed by quantification of the amount released. These approaches therefore do not allow direct real-time visualization at the micrometer length scale, which is mandatory for a detailed mechanistic description. We hypothesize that models characterizing and visualizing in real-time the transport processes of peptide and proteins in a matrix mimicking the subcutaneous tissue, *e.g.* hydrogels, may facilitate a better understanding of the transport mechanism and release in the subcutaneous tissue and potentially constitute a better platform for *in vitro* release testing. Hydrogel matrixes have been proposed and studied as simple models for subcutaneous tissue [8–11], due to their resemblance with respect to the porous structure in a gel-like matrix [12]. Various analytical techniques

Abbreviations: AAS, atomic absorption spectroscopy; DLS, dynamic light scattering; FRAP, fluorescence recovery after photo bleaching; FTIR, Fourier transform infrared; SEC, size exclusion chromatography; SEM, scanning electron microscopy; TDA, Taylor dispersion analysis.

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have been employed to measure the diffusion coefficients of proteins in hydrogels. The techniques used include fluorescence recovery after photobleaching (FRAP) [13–15], NMR [16], holographic interferometry [17,18], spectrophotometric methods [19], refractive index methods [10], and epifluorescence microscopy measurements [20]. Fourier transform infrared (FTIR) spectroscopic imaging is another analytical technique that can provide detailed information on processes related to drug release [21], including diffusion coefficients [22–24]. Methods involving transient diffusion of a compound into a gel followed by quantification of the average amount of the compound in a pre-defined volume of gel have also been employed [25,26], but these approaches are not direct as they measure only the product of the diffusivity.

UV imaging is the ability to generate temporally resolved data in the form of images from absorbance of UV light [27,28]. UV imaging was recently introduced for the study of drug diffusivity and release of low-molecular-weight compounds in hydrogel matrixes [29–31]. A characteristic feature of UV imaging is the possibility to follow drug transport in the immediate vicinity (within μm to mm range) of the formulation and in real-time without the need for compound labeling. The simple data read-out and interpretation, according to Beer's law, makes the technique well suited for formulation laboratories. The overall aim of our current research is to develop an *in vitro* model capable of characterizing by direct imaging and differentiating between the performance of parenteral peptide and protein formulations intended for subcutaneous administration. A critical step in the administration and delivery of protein drugs may be to ensure that the biomolecule is in an active conformation upon release from the formulation. Aggregation and denaturation events may be detrimental to the efficacy of a protein drug [32–35]. In relation to this point it was found of interest to explore whether a UV imaging-based setup utilizing a hydrogel matrix mimicking subcutaneous tissue was sufficiently sensitive to detect and monitor differences in the self-association states of a protein. Hence, using insulin as a model compound, the objective of the present study was to develop a UV imaging *in vitro* method allowing the real-time visualization and characterization of insulin diffusivity and self-association properties in a hydrogel matrix mimicking the subcutaneous tissue. The diffusivity, self-association, and apparent size of insulin were further characterized by Taylor dispersion analysis (TDA) in order to substantiate the results obtained from UV imaging. It is shown that the self-association properties of insulin are reflected in the diffusion coefficients obtained in the hydrogel matrixes indicating that such a UV imaging based technique may be of potential utility for *in vitro* release testing.

2. Experimental

2.1. Materials and sample preparations

Agarose (type I, gel point (1.5% (w/v) agarose in water): 34.5–37.5 °C; sulfate content: ≤0.15%) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Amorphous recombinant human insulin (rDNA) was obtained from Millipore (Bredford, MA, USA). Actrapid® Penfill® (100 IU/ml Human insulin (rDNA) was obtained from Novo Nordisk (Bagsværd, Denmark). Sodium hydroxide and sodium dihydrogenphosphate monohydrate were obtained from Merck (Darmstadt, Germany).

Phosphate buffered solutions with a concentration of 0.067 M and 0.17 M at pH 7.4 and 3.0, respectively, with an ionic strength of 0.15 M were used. The phosphate buffered solutions were prepared by dissolving an appropriate amount of sodium dihydrogenphosphate monohydrate in deionized water, and pH was adjusted to 7.4 and 3.0 using 5 M NaOH and 6.85 M HCl, respectively.

Insulin stock solutions with a concentration of 1 mM were made by weighing an appropriate amount of amorphous human insulin and dissolving it in 0.01 M HCl or 20% (v/v) acetic acid (20% (v/v) of the total volume) followed by addition of phosphate buffer to volume.

Agarose hydrogels were prepared by suspending a weighed amount of agarose powder, corresponding to 1.5 and 0.5% (w/v), in phosphate buffer at pH 3.0 and 7.4, respectively, followed by heating of the agarose suspensions to 98 °C for approximately 20 min to dissolve the agarose. The agarose solutions (~310 μl) were transferred to quartz cells (8.0 mm × 1.0 mm × 38 mm ($H \times W \times L$)) (Starna Scientific Ltd., Hainault, Essex, UK), and the lid of the quarts cells were placed on top of the agarose solutions. The quartz cells containing the pre-gels were left at room temperature for at least 7 and 1 h to ensure complete gelation of the gel matrixes at pH 3.0 and 7.4, respectively.

Cryo-scanning electron microscopy (SEM) pictures of the gel matrixes were obtained as described by Alhede et al. [36] Cryo-SEM measurements were carried out on a Quanta 3D FEG (FEI™, Europe NanoPort, Eindhoven, Netherlands). The SEM was equipped with the Quorum Tech PT2000T Cryo-Stage for preparing and transferring the frozen samples into the SEM measuring area. A platinum sputter coater was build-in the Cryo-Stage preparation-chamber to be able to coat the sample with platinum before the measurement.

2.2. Methods

2.2.1. Zinc content

The zinc content in the used insulin batch was determined by atomic absorption spectrometry (AAS) and by inductively coupled plasma mass spectrometry (ICP-MS). AAS was performed on a Perkin Elmer AAnalyst 200 atomic absorption spectrophotometer (Perkin Elmer, Shelton, USA) equipped with a zinc hollow cathode lamp and a monochromator. The measurements were performed at a wavelength of 213.9 nm. The zinc content was determined both from the measured absorbance of insulin-zinc solutions by the aid of a calibration curve in a concentration range from 0.25 to 2.5 ppm zinc and by the standard-addition method by adding 0, 1, 2, and 3 ppm zinc, respectively, to the samples. The absorbance of each sample was measured twice.

ICP-MS was performed on a PerkinElmerSciex DRC-e ICP mass spectrometer (Perkin Elmer, SCIEX, Norwalk, CT, USA) equipped with a Micromist nebulizer and with a standard spraychamber V2 (Glass Expansion, Melbourne, Austarlia). The instrument was run and controlled by Elan Software Version 3.4 (Perkin Elmer). The nebulizer gas flow rate, plasma RF power and lens voltage were optimized on a multi-standard solution. The zinc content was determined using the standard–addition method by monitoring the intensity of the ^{66}Zn and ^{68}Zn isotope. The zinc isotopes were monitored with two replicates per run.

2.2.2. Size exclusion chromatography

Size exclusion chromatography (SEC) measurements were carried out on a Young Lin 9100 HPLC system consisting of a YL9101 vacuum degasser, YL9110 quaternary pump, YL9130 column compartment, and YL9160 PDA detector (Young Lin Instrument Co., Ltd., Korea). A HPLC Guard Cartridge system GFC-2000 4 mm × 3.0 mm internal diameter (id) (Phenomenex, Torrance, CA, USA) was placed in front of a Shodex silica gel KW – 802.5 column (8 mm id × 300 mm) (Shodex, Japan). Sample volumes of 9 and 30 μl were injected onto the column, and the separations were performed with a flow rate of 0.5 ml/min. Phosphate buffered solutions with a pH of 7.4 and 3.0 were used as eluents. UV detection was performed at 214 or 280 nm.