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Note

Secondary structure alterations in insulin and growth hormone water-in-oil emulsions

Lene Jørgensen [a,*], Charlotte Vermehren [a], Simon Bjerregaard [b], Sven Froekjaer [a]

[a] *Department of Pharmaceutics, The Royal Danish School of Pharmacy, Universitetsparken 2, DK-2100 Copenhagen, Denmark*
[b] *Novo Nordisk, Pharmaceutics Ge, Hagedornsvej 1, DK-2820 Gentofte, Denmark*

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Abstract

Water-in-oil (w/o) emulsions have shown a promising release profile of small drug molecules and proteins. However, the major concerns are the structural stability, the retention of the activity and to avoid unwanted immunological reactions caused by the changes in protein structure. In the present study, the secondary structure of insulin and growth hormone is investigated after manufacture of w/o emulsions, using Fourier transform infrared (FTIR) spectroscopy. Initial investigations indicate an altered distribution in the secondary structure elements, e.g. α-helix and β-sheet, measured by area overlap calculations. The changes are more pronounced for growth hormone than for insulin. The overlapping area is 0.93 ± 0.01 for the emulsion containing insulin manufactured at 0 °C and homogenised for 3 min, the corresponding value for growth hormone is 0.83 ± 0.01. The droplet size changes from 0.27 ± 0.04 μm in the blank w/o emulsion to 0.79 ± 0.13 and 0.66 ± 0.21 μm when insulin or growth hormone is incorporated into the w/o emulsions, respectively.

Keywords: w/o Emulsion; FTIR; Insulin growth hormone; Secondary structure

Water-in-oil (w/o) emulsions can be used as depot formulation to provide a controlled release of a drug following intramuscular (i.m.) administration. Emulsions are flexible systems, to which release properties can be adjusted by several parameters such as volume fraction of the disperse phase, droplet size and osmotic gradient. A limiting or controlling factor in the release of drug from an emulsion may be the physical stability of the emulsion (Windheuser et al., 1970). It is, therefore, essential that emulsions are stable, as drug release can be affected by the physical changes of the emulsions. Furthermore, it is important that the drug substance, e.g. protein incorporated, maintains an active structure.

Several different aspects influence the physical stability of proteins. The changes in the structures that being secondary, tertiary, and quaternary caused by the instability might result in changed biological and pharmacological activity (Baudys and Kim, 2000) or unwanted immunological reactions (Cleland et al., 1993; Fu et al., 1999; Baudys and Kim, 2000). A frequently used method for studying structural changes is Fourier transform infrared (FTIR) spectroscopy (Haris and Severcan, 1999).

The aim of this study is to investigate the effects of manufacturing temperature and homogenisation time on changes in the secondary structure of the protein in the emulsion.

* Corresponding author. Tel.: +45-35306369.
E-mail address: lej@dfh.dk (L. Jørgensen).

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The w/o emulsions were prepared according to the procedure described by Bjerregaard et al. (Bjerregaard et al., 1999) with an aqueous phase of 60%, w/w. Fractionated coconut oil (Viscoleo), a medium chain triglyceride-oil (MCT-oil) supplied by H. Lundbeck A/S (Denmark), was used as oil phase containing 3.5%, w/w Polyglycerol polyricinolate (PG PR) kindly donated by Danisco Ingredients (Denmark) and 1%, w/w SPAN 80 (sorbitan monooleate) from Sigma Chemical Co. (St. Louis, MO, USA). The aqueous phase containing protein (20 mg/ml) was added dropwise to the oil phase containing the surfactants during agitation with a magnetic stirrer. Both insulin and growth hormone were kindly donated by Novo Nordisk A/S (Denmark) and used without further purification. Emulsification was achieved with Ultra Turrax T-25 (Janke and Kunkel, Germany) equipped with dispersing element S25N-25G at 13,600 rpm for either 3 or 5 min, the acquired temperature (0 and 25 °C) was achieved with ice- or water-baths.

The mean droplet size was determined by photon correlation spectroscopy (PCS) within the first hour of manufacture. The PCS system consisted of a Malvern Zetasizer 4 (Malvern Ltd., UK) with a helium–neon laser (wavelength = 633 nm) and a 7032 multi-8 correlator connected to a computer running Malvern PCS-software version 1.35 for data collection. About 1–3 μl of emulsion were dispersed in 1 ml Isopropylmyristat from Sigma. The Isopropylmyristat (viscosity: 6.5 mPa s, refractive index: 1.43) was saturated with water for 24 h prior to use. Measurements were carried out at 25 °C at a scattering angle of 90° in a glass cuvet. All sizes given are *z*-average-mean values.

IR-spectra were measured using a Bomem IR-spectrometer (Bomem, Canada). The samples were placed in a cell with CaF_2 crystal windows, and the light way was adjusted to similar path lengths in the different samples. For each spectrum a 256-scan interferogram was collected in single-beam with a 4 cm^{-1} resolution at 25 °C. Protein spectra were obtained according to the double subtraction procedure described by Dong et al. (Dong et al., 1990, 2000) to obtain a straight baseline in the region from 2000 to 1700 cm^{-1}. The spectra of the phosphate buffer and the oil-surfactant mixture were subtracted from the spectra of the protein emulsion separately. The second derivative spectra were obtained with a seven-point Savitsky-Golay derivative function and the baseline was corrected using a 2- to 4-point adjustment. In addition, the spectra were area-normalised in the amide I region from 1705 to 1595 cm^{-1} using the Bgrams software (Galactic Industries, Salem, NH). The spectra were analysed by area overlap according to Kendrick et al. (Kendrick et al., 1996).

Investigations indicated that the secondary structure of insulin and growth hormone (Figs. 1 and 2) measured by FTIR was affected by the manufacture of the emulsion. Changes in the content of α-helix (1648–1660 cm^{-1}) and β-sheet (1625–1640 and 1675–1695 cm^{-1}) are seen for both proteins when compared to the protein solution spectra. However, the changes are larger for growth hormone than for insulin.

No additional change was seen in the overlapping area of the insulin spectra with the insulin emulsion spectra when the emulsion was manufactured under different conditions. The overlapping areas of the solution and emulsion spectra are 0.93 ± 0.01 (0 °C, 3 min homogenisation), 0.92 ± 0.01 (0 °C, 5 min homogenisation), 0.94 ± 0.01 (25 °C, 3 min homogenisation) and 0.93 ± 0.01 (25 °C, 5 min homogenisation) ($n = 3$). A statistical analysis shows no significant difference between the different treatments.

More elaborate changes were observed between the different manufacturing conditions for the growth hormone emulsions (Fig. 2). The overlapping areas of the solution and emulsion spectra are 0.83 ± 0.01 (0 °C, 3 min homogenisation), 0.79 ± 0.03 (0 °C, 5 min homogenisation), 0.85 ± 0.02 (25 °C, 3 min homogenisation) and 0.83 ± 0.01 (25 °C, 5 min homogenisation) ($n = 3$). A statistical analysis shows no significant difference between the different treatments.

The droplet size changes from 0.27 ± 0.04 μm ($n = 4$) in the blank emulsion to 0.79 ± 0.13 μm ($n = 11$) and 0.66 ± 0.21 μm ($n = 6$) when insulin or growth hormone is incorporated into the w/o emulsions, respectively. There is no immediate indications of any change in stability of the w/o emulsions caused by the incorporation of protein.

When proteins are exposed to interfaces their structure is very likely to change and, e.g. result in increased aggregation tendency (Norde and Lyklema, 1991). Katakam et al. (1995) and Hagenlocher and Pearlman (1989) observed that growth hormone aggregates when exposed to the air–solution interface during agitation. Similar effects are seen by Sluzky et al. (1991)

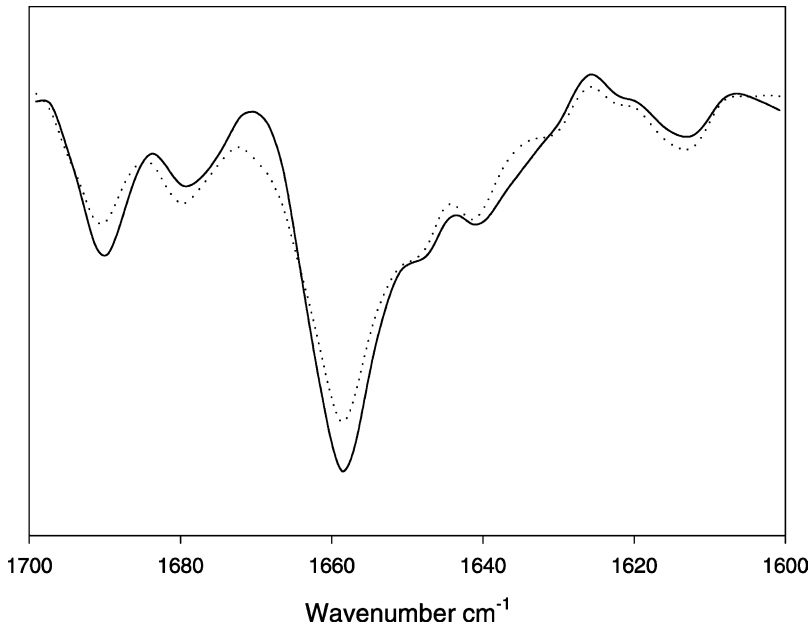

Fig. 1. shows the FTIR spectra of the insulin solution (20 mg/ml) in phosphate buffer 10 mM pH 7.4 (continued line) and w/o emulsion containing insulin (dotted line). The emulsion is manufactured at 0 °C and homogenised for 3 min. The overlapping area of the insulin emulsion spectra compared to the insulin solution spectra is 0.93 ± 0.01 ($n = 3$).

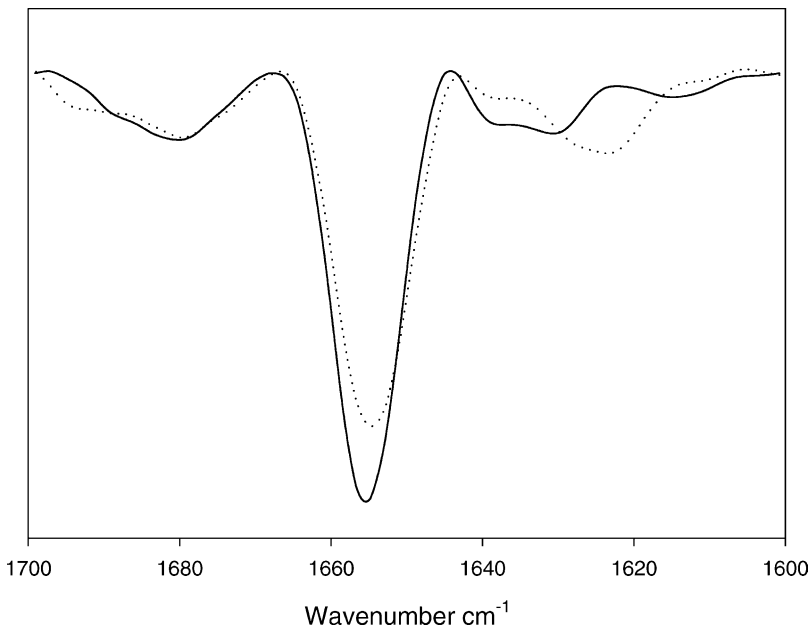

Fig. 2. shows the FTIR spectra of the growth hormone solution (20 mg/ml) in phosphate buffer 10 mM pH 7.4 (continued line) and w/o emulsion containing growth hormone (dotted line). The emulsion is manufactured at 0 °C and homogenised for 3 min. The overlapping area of the growth hormone emulsion spectra compared to the growth hormone solution spectra is 0.83 ± 0.01 ($n = 3$).

and Brange and Langkjaer (1993) for insulin when exposed to agitation. Results presented here show no similar tendency where severe aggregation is not seen even when the protein is exposed to mechanical stress and the oil–water interface. This might indicate that the exposure to the oil–water interface combined with the mechanical stress of manufacture is not quite as harsh a treatment as simple shaking and exposure to the air–solution interface. The possible explanations might be protection of the protein from exposure to interfaces by the added surfactants (Bam et al., 1998).

Several investigations of the secondary structure of protein when exposed to the organic–water interface in the manufacture of microspheres have been published lately (Cleland et al., 1997; Fu et al., 1999; van de Weert et al., 2000). The most prevailing tendency is that the secondary structure is changed considerably but it is possible to obtain a formulation without changes in the protein structure (Cleland et al., 1997). When compared to the changes in the secondary structure presented here, the w/o emulsion drug delivery system also shows considerable promise by largely preserving the secondary structure of the proteins during and after manufacturing.

In order to attain additional information about the w/o emulsion as a drug delivery system for proteins, attention need to be paid to the effect the incorporated protein might have on the release characteristic from the w/o emulsion. In addition, the question of preserved activity of the incorporated protein also needs to be addressed.

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