Study of the Aggregation of Insulin Glargine by Light Scattering

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ABSTRACT: Insulin glargine (Lantus®, Aventis Pharma, Deutschland, GmbH) is a new long-acting human insulin analog. Structural modification of the insulin molecule at two sites alters its pH, causing insulin glargine to precipitate in the neutral environment of subcutaneous tissue and to form a depot that is slowly absorbed into the bloodstream. In this paper insulin glargine aggregation is investigated by light scattering. This study shows that, in a physiologic-like pH (even at low ionic strength) conditions, aggregation phenomena occur, giving rise to compact structures with radius of hundreds of nanometers. The aggregation of insulin glargine can be responsible for its slow *in situ* absorption allowing for a more controlled release. J Pharm Sci 95:1029–1034, 2006

Keywords: HOE 901; insulin glargine; light scattering; protein aggregation; microscopy; particle sizing; solubility; colloid

INTRODUCTION

Diabetes Mellitus consists of a group of metabolic diseases characterized by hyperglycemia resulting from a relative or absolute deficiency of the hormone insulin. Type 1 diabetes is caused by an absolute deficiency in insulin secretion, Type 2 diabetes, instead, is caused by insulin resistance combined with inadequate compensatory insulin secretion.[1–3]

One of the goals of insulin treatment is to attain normal glycemia by maintaining appropriate insulin concentrations throughout each 24-h period. Intermediate- and long-acting insulins have been developed for once-daily administration through complexing with protamine or zinc to delay absorption. However, these products are associated with excessive rates of hypoglycemia because of pronounced peaks after injection and a duration of action that is too short to maintain glycemic control with once-daily injection, or high variability of absorption.[4]

Insulin glargine (Lantus®, Aventis Pharma, Deutschland, GmbH) is a new long-acting human insulin analog which represents a promising new alternative for basal insulin.

It was approved for use in patient with Type 1 and Type 2 Diabetes Mellitus by the US Food and Drug Administration in April 2000 and by the European Agency for the Evaluation of Medicinal Products in June 2000. Recombinant DNA technology, using a nondisease-producing laboratory strain of *Escherichia coli* (K12) plasmid DNA, has permitted the design of insulin glargine (HOE 901, 21A-Gly-30Ba-L-Arg-30Bb-L-Arg-human insulin) whose structural formula is shown in Figure 1.

Insulin glargine has a molecular weight of 6063 and its molecular formula is $C_{267}H_{404}N_{72}O_{78}S_6$; it results from two modifications of human insulin.

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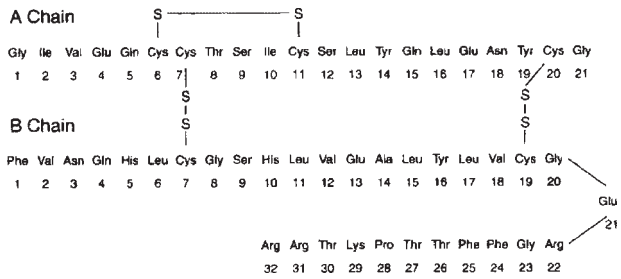

Figure 1. Structural formula of insulin glargine.

The first is the addition of two positive charges (two arginine molecules) to the C-terminus of the B-chain, which shifts the isoelectric point from a pH of 5.4–6.7, making the molecule more soluble at a slightly acidic pH and less soluble at the physiological pH of subcutaneous tissue. Because the derivative is formulated at an acidic pH of 4.0 (which means that it cannot be mixed with insulin prepared at a neutral pH, such as regular insulin), a second modification is needed to prevent deamidation and dimerization *via* the acid-sensitive asparagine residue at position 21 in the A-chain. The replacement of A21 asparagine by glycine is charge neutral and associated with good stability of the resulting human insulin analog. Studies of insulin glargine in animals have indicated a protracted action profile compared with Neutral Protamine Hagedorn (NPH) insulin;[5–7] studies in healthy human subjects have indicated a flat insulin profile with no peaks, in contrast to the early insulin peak and return to pre-injection values observed with NPH insulin.[8] Early clinical trials in patients with Type 1 Diabetes Mellitus indicated the suitability of insulin glargine for use as basal insulin.[9]

When injected subcutaneously, insulin glargine, which is a clear solution, forms a microprecipitate at the physiological, neutral pH of the subcutaneous space. The formation of microprecipitates delays the absorption of insulin glargine from the injection site and extends its duration of action.

In crystallography studies, an increase of the intramolecular bonding of the insulin hexamer was observed.[10] When the insulin hexamer and higher aggregates are stabilized, the nature of the precipitate and the rate of its dissolution and absorption from the injection site are affected. Insulin glargine, therefore, has a delayed and prolonged absorption from the subcutaneous injection site.[4]

The drug has a 24-h basal insulin profile with almost no peak in activity. Insulin glargine is generally administered once daily as a subcutaneous injection at bedtime, in a regimen that includes premeal regular insulin or insulin lispro. However, when used in low doses, as in pediatric patients, two injections may be needed for 24-h coverage. For a better understanding of the pharmacokinetic and pharmacodynamics of insulin glargine, the knowledge of microscopic processes involved in the insulin crystal growth is required. In this direction, the first step was made by Mühlig et al.[11,12] who used the confocal laser scanning microscopy (CLSM) to study the step kinetics in protein crystal growth and showed that this technique is an efficient tool to observe protein crystallization.

The aim of the present work is to investigate insulin glargine solutions, by monitoring its aggregation by light scattering just after the preparation of the solution. This method allows for the determination of the nanoaggregate size in a physiologic-like pH and at different concentration and temperature values. The aggregates are also observed by microscopy when their size reaches micrometric values.

EXPERIMENTAL

Materials

For the experiments the commercial formulation of insulin glargine Lantus®, kindly offered by Aventis Pharma, was used. In this formulation, insulin glargine is dissolved in a clear aqueous fluid and is available in a concentration of 3.6 mg/mL. Each milliliter of insulin glargine also contains 30 μg of zinc, 2.7 mg of m-cresol, 20 mg of glycerol 85%, and water for injection. Commercial analytical grade zinc chloride was obtained from Carlo Erba (Milano, Italy); water for injection from Angelini (Roma, Italy).

Sample Preparation

Solutions of insulin glargine were prepared by diluting the initial formulation with water for injection and then adjusting pH with the addition of the proper quantity of a 100 mM NaOH aqueous solution. The pH value of the final solutions was measured with the pH-meter of the ZetaPALS instrument by Brookhaven Instruments Corporation, which was calibrated by using the ZetaPALS software and three standards at pH = 4, pH = 7, and pH = 10 (purchased by Sigma-Aldrich, Milano, Italy) at pH = 298 K. The pH of

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all the final diluted solutions of insulin glargine was 7 ± 0.2.

In order to ensure that different concentrations of NaOH do not affect the final aggregate size, the ionic strength of the solution was increased up to 0.1% by weight adding NaCl. No significant changes were observed.

No filtration procedure was done on the samples and all the measurements were replicated three times. The temperature was varied in the range 295–310 K.

Solutions of regular insulin was prepared by diluting the pharmaceutical formulation (by Eli Lilly Italia, Firenze, Italy) and adjusting pH to 7 as described above; the acidic solution of regular insulin at pH = 4 was obtained by adding the proper amount of a 100 mM HCl aqueous solution.

Data Analysis

Static and dynamic light scattering experiments were carried out by using a He–Ne laser source (15 mW) and a computerized homemade goniometer which allowed for collecting the scattered light at different scattering angles between 30 and 140°.

For the dynamic light scattering measurements the scattered signal was sent to a Malvern 4700 correlator to obtain the normalized intensity autocorrelation function:[13–15]

$$g_2(Q,t) = \frac{\langle I(Q,0)I(Q,t)\rangle}{\langle I(Q,0)\rangle^2} = 1 + \alpha|g_1(Q,t)|^2 \quad (1)$$

where $Q = (4\pi n/\lambda_0)\sin(\theta/2)$ is the exchanged wave vector (θ being the scattering angle, n the refractive index of the solution, λ_0 the wavelength of light in vacuum and α a spatial coherence factor) and $g_1(Q,t) = \langle E^*(Q,0)E(Q,t)\rangle / \langle I(Q,0)\rangle$ the normalized scattered electric field correlation function. The right hand of Eq. 1 holds provided that, as in our case, the scattered electric fields obeys a Gaussian statistics (Siegert's relation).

In the case of dilute solutions of monodisperse particles the scattered electric field correlation function can be written as $g_1(Q,t) = \exp(-DQ^2t)$, where D is the translational diffusion coefficient of suspended particles. By using the Einstein–Stokes relation, the hydrodynamic radius, R_H, can be calculated: $R_H = k_B T/(6\pi\eta D)$, where k_B is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of the solvent. Actually, only ideal solutions are monodisperse; real systems, and colloids in particular, have a size polydispersity. In this case, the correlation function may be expressed as the Laplace transform of a continuous distribution $A(\tau)$ of decay times ($\tau = (DQ^2)^{-1}$):

$$g_1(t) = \int \tau A(\tau) \exp(-t/\tau) \mathrm{d}(\ln \tau) \quad (2)$$

with the mean relaxation time $\langle\tau\rangle = \int \tau \mathrm{A}(\tau) \mathrm{d}\tau$.

To perform this inversion procedure a discrete multiexponential nonnegative least-squares fit (NNLS) was used.

The static light scattering measurements consist in collecting the intensity at different scattering angles; the intensity scattered by solutions, after subtraction of that of the solvent, is normalized by the intensity of toluene used as reference. The so-obtained profile can be represented by the following relation:[13,15,16]

$$I(Q) = NKM_W cP(Q)S(Q) \quad (3)$$

in which $P(Q)$ and $S(Q)$ are the normalized form factor and the structure factor, respectively, N the aggregation number, M_W the molecular weight of the insulin glargine, c the mass concentration and K the optical constant (for the scattering geometry[17] of this experiment is $K = \frac{4\pi^2 n^2}{\lambda_0^4 N_A}\left(\frac{dn}{dc}\right)^2$). In the case of the insulin glargine solutions studied in the paper interaction between particles can be neglected and $S(Q) \approx 1$.

RESULTS AND DISCUSSION

Figure 2 shows that the correlation function of the commercial formulation displays two relaxation

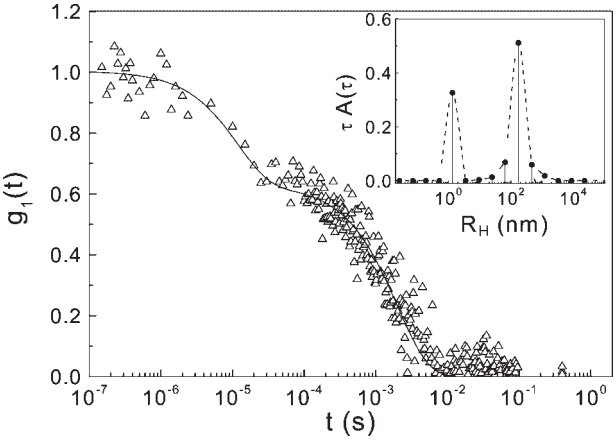

Figure 2. Normalized correlation function of the commercial formulation at a scattering angle of 90°; the continuous line is the fit according to the NNLS inversion. The inset reports the size distribution as obtained by the inversion method.

modes, indicating that solution contains mainly two families of particles with different size. The NNLS inversion (Eq. 2) allows for obtaining the size distribution of particles, as reported in the inset of Figure 2: the average hydrodynamic radius of the smaller particles is about 2 nm and that of the larger ones is about 200 nm. The first component can represent the diffusion of insulin glargine, but the second component indicates the presence of aggregates. However, these aggregated forms of insulin are present at very low concentration. In fact, because the scattered intensity is dependent of the particle's mass, by correcting the distribution according to the Mie's theory, it can be calculated that more than 99.9% of the total mass is distributed in the smaller particles. Therefore, in the acidic environment of the commercial formulation, insulin glargine is in its oligomeric form. By considering that the form factor of the smaller particles (much smaller than the wavelength of light) at 90° is identifiable with the value at $Q = 0$ ($P(0) = 1$), whereas the form factor of the large aggregates is less than one at 90°,[15,16] it is possible to estimate the average aggregation number of the oligomers by Eq. (3). It is estimated $N \approx 11$, about twice the value of the hexameric form of insulin; this result is due to the fact that the average of the aggregation number is weighted by the scattered intensity and about half of the total intensity at 90° is scattered by aggregates. Therefore, it is likely that the oligomeric form of insulin glargine is actually constituted by hexamers.

By diluting the formulation and varying pH to the physiological value, the aggregation process is favored for concentrations above 0.01 mg/mL, which represents a critical aggregation concentration; the presence of this concentration threshold makes insulin glargine solution analogous to colloidal systems.

As summarized in Table 1, the solution at a concentration of 1.8 mg/mL appears fully clustered with aggregates having micrometric size, also visible by means of optical microscopy (see Fig. 3). On decreasing concentration significantly to about 0.1 mg/mL, aggregate hydrodynamic radius decreases too, but remains constant on further dilution at a value of about 180 nm. Although the radius of the aggregates is comparable with that obtained for the initial formulation, the number of aggregates is noteworthy larger and the corresponding contribution to the scattering becomes predominant. In this case, the intensity profile can give information on the form factor of the aggregates. Figure 4 shows the scattered intensity of the solution at $c = 0.05$ mg/mL, as example, along with the fit obtained by modeling aggregates as homogeneous polydisperse spheres:

Table 1. Hydrodynamic Radius Values as a Function of Concentration at pH = 7

c (mg/mL)	R_H (nm)
1.8	1800 ± 200
0.1	140 ± 30
0.07	180 ± 30
0.05	180 ± 30
0.025	160 ± 30

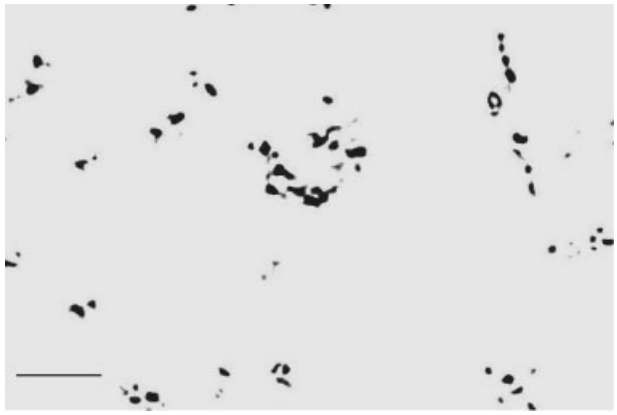

Figure 3. Image of the insulin glargine aggregates in solution ($c = 1.8$ mg/mL at pH = 7) obtained by an inverted Zeiss microscope (Axiovert S100 with an objective 63X NA = 1.25). The solid line represents 10 μm.

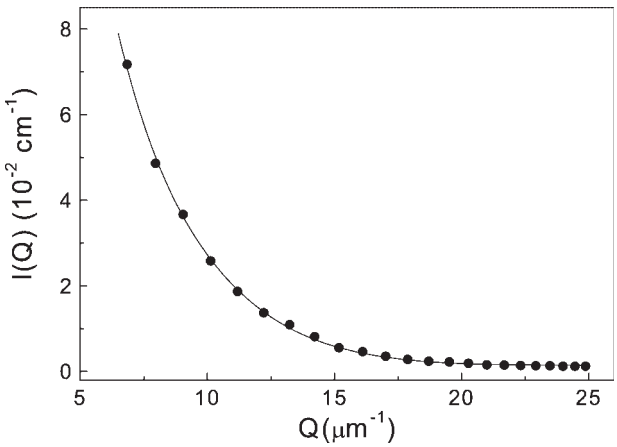

Figure 4. Scattered intensity profile of the solution at $c = 0.05$ mg/mL (pH = 7). The continuous line is the fit according to the homogeneous sphere model.

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$$P(Q)=\int\left[3\frac{\sin(QR(x))-QR(x)\cos(QR(x))}{(QR(x))^3}\right]^2 f(x)\mathrm{d}x \quad (4)$$

where $f(x)$ is the Gaussian distribution around the mean radius R.

The obtained value of the radius is $R=350$ nm with a polydispersity of about 50%. This high polydispersity is responsible for the difference between the results from dynamic and static scattering.[18]

The fitting procedure allows for the determination of the intensity at $Q=0$, which, according to Eq. (3), is related to the aggregation number, N. The latter is about 10^6 molecules.

Moreover, the increase of the ionic strength (to about 0.1% by adding NaCl) did not affect the system significantly.

In order to understand if the aggregation process is fostered by the decreased amount of zinc (because of the dilution procedure), which is known to stabilize the insulin hexamers,[19,20] a solution of zinc chloride containing the same zinc concentration as the commercial formulation has been used to dilute insulin to 0.05 mg/mL (pH is adjusted to 7 according to the procedure described in the experimental section). Figure 5a shows that within 2 h in both solutions with and without zinc chloride aggregates take the same size (200 ± 30 nm and 180 ± 30 nm, respectively), whereas thereafter in the solution without salt aggregate hydrodynamic radius increases progressively and after 3 h becomes about 700 ± 100 nm. The sample with added $ZnCl_2$, instead, remains unchanged. A tentative explanation of these findings could be ascribed to a different conformation (size and charge of the building blocks) of the aggregates, that, at pH = 7 in the absence of zinc, causes colloidal instability.

After reaching the metastable equilibrium (mean radius of 700 nm), the effect of temperature on the aggregates was checked. On increasing temperature up to 310 K, and maintaining the solution at the selected temperature for 8 h, no changes were observed either in the scattered intensity or in aggregate size.

As comparison, the same scattering approach has been performed for aqueous solutions of regular insulin, both at neutral and acidic pH; there is no evidence of extended aggregation in these solutions, indicating that regular insulin is present, at most, in its oligomeric form (hexamer). In fact, as it is known, the lower isoelectric point of the regular insulin guarantees a sufficient surface charge to prevent extended aggregation.

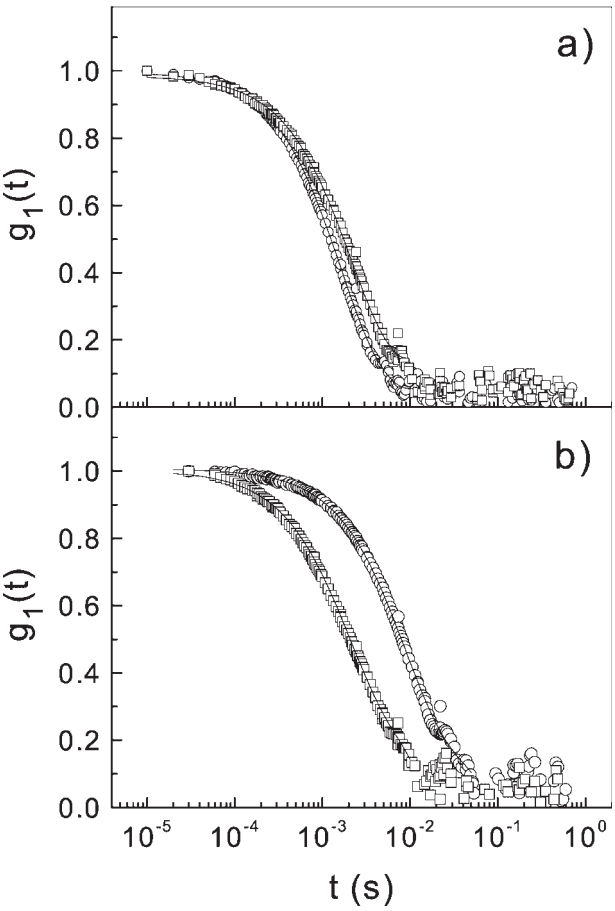

Figure 5. Comparison between the correlation functions of the fresh (plot a) and aged (plot b) solution at $c=0.05$ mg/mL with (squares) and without (circles) added $ZnCl_2$ (pH = 7).

CONCLUSIONS

Unlike regular insulin, insulin glargine molecules undergo aggregation in neutral environment. The results of this study show, through light scattering techniques, that the commercial formulation of insulin glargine contains mainly the oligomeric form of the molecule together with a very small number of nanoaggregates. In a neutral environment, mimicking the physiologic pH, insulin glargine rapidly aggregates; at the highest concentration aggregates have micrometric size (as also visible by means of microscopy) and at lower concentration solution is mainly constituted by

aggregates with radius of few hundreds of nanometers.

These findings show that the extended aggregation phenomena can be responsible for the slow *in situ* absorption of insulin glargine allowing for a more controlled release.

REFERENCES

1. Kahn CR, Shechter Y. 1990. Insulin, oral hypoglycemic agents, and the pharmacology of the endocrine pancreas. In: Rall TW, Nies AS, Taylor P, Gilman AG, Goodman LS, editors. The pharmacological basic of therapeutics, 8th edition, New York: Pergamon press, pp 1463–1495.
2. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. 2000. Diabetes Care 23:S4–S19.
3. Campbell RK, White JR, Levien T, Baker D. 2001. Insulin glargine. Clin Ther 23:1938–1957.
4. Investigation Group of Aventis Pharmaceuticals. 2003. Safety and efficacy of insulin glargine (HOE 901) versus NPH insulin in combination with oral treatment in Type 2 diabetic patients. Diabetic Medicina 20:545–551.
5. Seipke G, Geisen K, Neubauer HP, Pittius C, Rosskamp R, Schwabe D. 1992. New insulin preparations with prolonged action profiles: A21-modified arginine insulins. Diabetologia 35:A4.
6. Seipke G, Berchthold H, Geisen K, Hilgenfeld R, Rosskamp R. 1995. HOE 901: A new insulin with prolonged action. Eur J Endocrinol 132:25.
7. Bolli GB, Owens DR. 2000. Insulin glargine. The Lancet 356:443–445.
8. Coates PA, Mukherjee S, Luzio S, Srodzinski KA, Kurzhals R, Rosskamp R, Owens DR. 1995. Pharmacokinetics of a "long-acting" human insulin analogue (HOE 901) in healthy subjects. Diabetes 44:130A.
9. Talaulicar M, Willms B, Rosskamp R. 1995. Efficacy of HOE 901 following subcutaneous injection for four days in Type 1 diabetic subjects. Diabetologia 37:A169.
10. Hilgenfeld R, Dorschug M, Geisen K, Neubauer H, Obermeier R, Seipke G, Berchtold H. 1992. Controlling insulin bioavailability by crystal contact engineering. Diabetologia 35:A193.
11. Mühlig P, Klupsch Th, Schell U, Hilgenfeld R. 2001. Observation of the early stage of insulin crystallization by confocal laser scanning microscopy. J Crystal Growth 232:93–101.
12. Mühlig P, Klupsch Th, Kaulmann U, Hilgenfeld R. 2003. Noninvasive in situ observation of the crystallization kinetics of biological macromolecules by confocal laser scanning microscopy. J Struct Biol 142:47–55.
13. Berne BJ, Pecora R. 1976. Dynamic Light Scattering with application to chemistry, biology and physics. New York: Wiley-Interscience.
14. Brown W, Nicolai T. 1993. Dynamic properties of polymer solutions. In: Brown W, editor. Dynamic Light Scattering. Oxford: Clarendon, pp 272–318.
15. Chu B. 1991. Laser Light Scattering-Basic Principle and Practice, 2nd edition, San Diego: Academic.
16. Mallamace F, Micali N. 1996. Low angle light scattering and its applications. In: Brown W, editor. Light Scattering: Principles and Development. Oxford: Clarendon, pp 381–438.
17. The value of the differential refractive index dn/dc is set equal to 0.18, typical of protein aqueous solutions.
18. Pencer J, Hallett FR. 2003. Effect of vesicle size and shape on Static and Dynamic Light Scattering measurements. Langmuir 19:7488–7497.
19. Hill CP, Dauter Z, Dodson EJ, Dodson GG, Dunn MF. 1991. X-ray Structure of an Unusual Ca^{2+} Site and the Roles of Zn^{2+} and Ca^{2+} in the Assembly, Stability, and Storage of the Insulin Hexamer. Biochemistry 30:917–924.
20. Liu F, Kildsig DO, Mitra AK. 1991. Insulin aggregation in aqueous media and its effect on alpha-chymotrypsin-mediated proteolytic degradation. Pharm Res 8:925–929.