In Vitro Dissolution of Insulin Crystal Polymorphs at Model Conditions Relevant to In Vivo Environment

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ABSTRACT

In vivo dissolution of crystalline insulin formulations is an important step for insulin absorption in the subcutaneous therapy of diabetes, which is the most widespread. Here, data on the in vitro dissolution of three different crystalline forms of commercial porcine insulin are reported. The aim of this study was to create a model framework of in vivo dissolution of insulin crystalline formulations.

In situ insulin crystal dissolution was studied at conditions of continuous undersaturation (i.e., in the absence of any free insulin around the crystals) in several types of model flood liquids, at different flooding flow rates relevant to blood capillary flows, and at a dissolution system temperature of 36 °C.

Insulin crystal dissolution rate depends strongly on the flooding liquid composition and crystal type, the effects of pH and flooding flow rate being less pronounced. Results of the present study suggest a possibility for purposeful control over in vivo dissolution rates through development of particular insulin crystalline-based formulations.

KEYWORDS: Dissolution, insulin crystal polymorphs, diabetes therapy, insulin crystalline-based formulations, physiological conditions.

INTRODUCTION

Insulin is a protein hormone of key importance for glucose and fat metabolism. A deficiency or resistance to insulin results in the development of a metabolic disorder called diabetes mellitus, which includes several specific disease types. As this is now an extremely widespread disorder, insulin has been the object of intensive investigation for several decades. Accordingly, plenty of therapeutic strategies have been developed, as the major forms of diabetes are chronic conditions and cannot be cured practically. Known insulin therapeutics include mainly various insulin suspensions and formulations based on the protein amino acid variation for subcutaneous injection. Different strategies are used when a rapid, intermediate, or long-acting version of the medicine is desired. In some emergency cases, solutions of insulin can be infused intravenously. Although some inhalable insulin formulations were developed a few years ago and presently much effort is being made on orally administered insulin drugs (1), the injectable formulations remain the most used and popular insulin source for diabetes therapy. A comprehensive review of insulin formulations and delivery systems is given elsewhere (2).

The main issue in supplying exogenous insulin to a body with diabetes mellitus concerns time variations in the drug uptake. This is reflected in the time for the dissolution of insulin particles and in insulin diffusion into the blood-stream upon subcutaneous injection. Generally, crystalline suspensions containing zinc are used when prolonged insulin action is required. Zinc is indispensable for hexameric insulin formation and strongly affects hexamer–monomer conversion rate (3). Both experimental and theoretical studies have shown two rate-limiting steps for the dissolution of zinc–insulin suspensions, namely surface reaction, when zinc-extracting compounds were used, and the diffusion of insulin species into solution (4, 5). Our previous results also show that the dissolution rate of zinc-containing rhombic insulin crystals was strongly affected when crystals were flooded by zinc-rich solution (6). The aim of the present investigation was to shed light on the dissolution of different crystal polymorphic forms of insulin at conditions relevant to the physiological environment.

EXPERIMENTAL

Commercial insulin from porcine pancreas (BioChemika, ≥85%, =24 IU/mg) was used for all crystallization experiments. Insulin crystal nucleation, crystal growth, and subsequent crystal dissolution were studied in a thermoregulated glass chamber system (Figure 1). It consisted of a quasi-two-dimensional glass cell (known as Hele–Shaw cell configuration) integrated in a glass jacket. The reduced crystallization glass cell height was 100 μm and the diameter was 30 mm. This construction resulted in a small internal cell volume and low protein consumption. The chamber transparency ensured perfect microscopic observation, undistorted imaging with an eyepiece camera,
and real-time observation of crystal growth and the dissolution processes. The whole chamber was connected to a thermostat for precise temperature regulation. To mimic physiological temperature conditions, most of the crystal growth and dissolution studies were performed at 36 °C. A closed flood solution reservoir system (Mariotte’s bottle) was used with an air tube bubbler, again integrated in the thermo-jacket and mounted in the path of the tempering water bath. The reservoir solution was used to flood insulin crystal populations during the dissolution studies. The solution flow was driven by hydrostatic pressure and tuned to a desired flow rate by a micro-screw system connected to the reservoir chamber. The pressure was kept constant in time because of the bubbler system. Although there should be a slight variation of that pressure each time a new air bubble escapes from the dip end of the tube, the effect was generally very small and resulted in negligible flow oscillation.

**Monoclinic Insulin Crystals**

To obtain monoclinic insulin crystals, crystallization screening was performed using insulin concentrations increasing in small increments over the interval 1.9–9 mg/mL. In all screening experiments, a considerable population of microscopic insulin aggregates formed immediately upon solution component mixing and disappeared completely at room temperature for a period of time that was proportional to the concentration of insulin in the system (ranging from a few to several hours). About four days were needed to obtain microscopically visible monoclinic crystals.

Further screening with respect to the temperature was made to find appropriate conditions for the growth of stable monoclinic crystal populations at physiological temperature (36 °C). Even at relatively high (for common protein crystallization) insulin concentrations (e.g., 13 mg/mL), no crystal nucleation was observed at 36 °C for up to three days after very fast dissolution of initial aggregates (about 30 min). That suggests a strong dependence of insulin solubility on temperature at this particular crystallization condition. A temperature of 15–20 °C provided for crystal nucleation, and at 36 °C, no crystal dissolution was observed. With even higher insulin concentrations (16.7–18.1 mg/mL), a good balance among fast initial aggregate dissolution (about 1–2 h), crystal nucleation (in 2 h), and crystal growth (no more than 24 h) was found. Large regular monoclinic crystals obtained in this manner were used for the subsequent dissolution studies. All of the crystallization mixture constituents (1% phenol, 0.05 M sodium citrate, 0.04% Zn ions as zinc chloride) were added to the insulin powder directly as a premixed solution at pH 6.89.

**Cubic Insulin Crystals**

Cubic insulin crystals were grown in a solution of 0.01 M sodium acetate, 0.7% sodium chloride, and 0.1% methyl parahydroxybenzoate at pH 7.92. Again, this composition was added directly to the insulin powder to prevent considerable changes in mixture pH, which is more probable in stepwise solution preparation. These conditions imply a moderate temperature dependence of insulin solubility, probably because of the sodium chloride present in the crystallization solution. The crystallization system was subjected to extensive screening for optimization of crystal nucleation and growth conditions with respect to temperature. Generally, some aggregates were observed upon solution mixing. Therefore, a common practice was to use temperature cycles in the range of 10–60 °C for aggregate dissolution and crystal renucleation. Fresh nucleation and growth took as much as four days. The insulin concentrations were in the interval of 4.5–11 mg/mL because occasionally there was poor reproducibility among the crystallization trials, probably because of partial insulin denaturation at the highest temperatures used in the temperature cycles. The use of a flexible experimental setup, however, allowed for fine manipulation and produced stable crystal populations at 36 °C.

**Tetragonal Insulin Crystals**

Conditions for growing tetragonal insulin crystals were based on those used by Cutfield et al. (7). A final solution containing 5 mg/mL insulin, 0.004 M HCl, 0.004 M sodium acetate, and 16% (v/v) acetone at pH 4.51 was injected in the crystallization cell at 36 °C. Initially a light insulin precipitate was observed in the system. Crystal nucleation took place in a few hours, mainly in the precipitate-rich areas, which is a typical example of protein crystal growth from a light precipitate, until the precipitate disappeared completely in a day. To speed up the crystallization trials, somewhat lower temperatures could be used initially, as all of the crystal populations were stable at 36 °C at the given conditions. The grown crystals (Figure 2) represent a variety of shapes (e.g., hourglass, corner-sharing tetragonal pyramids, lemon-like).

**RESULTS AND DISCUSSION**

Three different model liquids, bidistilled water, saline solution (0.9% sodium chloride), and human blood
plasma, all equilibrated at 36 °C, were used for flooding the insulin crystal populations.

**Dissolution of Monoclinic Insulin Crystals**

In spite of unusually high insulin concentrations used for growing the monoclinic insulin crystals, a large population of relatively small insulin crystals failed to grow at a temperature of 36 °C. Large and perfectly shaped crystals formed, which were stable at that temperature. Although zinc stabilizes rhombohedral insulin crystals (5), monoclinic insulin crystals were extremely fragile in comparison to the other crystal forms investigated here when dissolution via undersaturation in a flow was attempted (Figure 3). In that case, the term crystal disintegration would be more appropriate.

The observed remarkable crystal fragility is a consequence of the high water content of the crystals (8) rather than a property of the crystal size itself. Figure 3 illustrates that monoclinic insulin crystal disintegration is not explicitly dependent on the flow rate, considering a difference of three orders of magnitude and crystal disintegration generally on the same time scale. The more surprising result is that in only bidistilled water (Figure 4), the crystals again disintegrated upon flooding with rates comparable to those in the saline flood solution (Figure 3). However, the pattern of disintegration seemed to be different. The saline flood solution led to disintegration that was more apparent than the inconspicuous one achieved with bidistilled water. These results imply that the dissolution (i.e., disintegration) rate of monoclinic insulin crystals depends neither on the flood solution composition (NaCl and bidistilled water) nor on the pH of the flood liquid (bidistilled water) in the mentioned interval. The modest dependence on the flow rate itself is a trivial effect, detectable in this particular case when significantly different flow rates were used (e.g., Figure 3).

On a nanoscale (e.g., insulin hexamers) after crystal disintegration, monoclinic insulin could be stable enough in the presence of phenol (9), which is an insulin aggregation agent (10). This, however, is not a reasonable expectation if the goal is a continuous decrease in compound concentration in the crystal vicinity to mimic the in vivo environment. In the present in vitro setup, this drop is almost immediate.

Because one of the primary goals was to model crystal dissolution behavior at physiological conditions, a study was performed using human blood plasma as a flood solution, keeping the whole system again at 36 °C. Furthermore, because subcutaneously injected insulin migrates upon absorption as an oligomeric species (11), it was reasonable to model crystal dissolution rates in the bloodstream. The blood plasma had the strongest positive effect on crystal dissolution rate, probably due to its bicarbonate content (12), although some controversial results were obtained with particular insulin preparations (13–15). The important observation here was that the monoclinic insulin form exhibited true dissolution behavior on a microscopic scale, resembling the melting of ice in water (Figure 5).

**Dissolution of Cubic Insulin Crystals**

Cubic insulin crystals represented the most versatile system in the present study. A positive artifact of the large number of temperature cycles applied was that after each cycle, nucleated crystals grew larger with more regular habit
In addition, a sharp reduction in crystal nucleation time was detected for high-numbered temperature cycles, perhaps in consequence of the existence of a stable population of insulin clusters after temperature-induced dissolution during each cycle. In some cases, several cycles were performed for about two weeks to obtain the desired crystal population. Treating the crystallization solution in such way provided the possibility to perform some dissolution experiments as a function of temperature as well (see below).

Figure 7 shows an attempt for dissolution of cubic insulin crystals in bidistilled water at pH 5.65, pH 7.00, and flow rates of several magnitudes. Note that at both pH values, there was practically no system response, which is why only one graph is shown. Any further consideration is irrelevant to the goals of the present study. Negligible microscopic dissolution was observed for about 24 h, rendering the dissolution process also independent of the flow rate. It is important to note that flood liquid flow rates used in the present study spanned the interval typical for the flow in blood capillaries. Small portions of the cubic crystals randomly detached during the dissolution experiments, which is evidence of poor crystal adhesion (i.e., loose interaction of crystal with the support or small contact areas) on the glass cell walls. The error bars in Figure 7 are instructive, as measurement during image analysis fell below reliable resolution. Figures 7–9 represent data from measurements of over 50 individual crystals in several distinct experiments.

More pronounced dissolution rates were detected when the cubic crystal population was flooded with 0.9% NaCl, pH 7.00, 36 °C, at a flow rate of 3.5 μL/sec, the cell being cleaned of crystals for about 15 min. No microscopically smooth dissolution was observed, the crystals detached much faster than in bidistilled water, and some portion partially disintegrated before detachment. A supporting study for measuring the dissolution rates at 15 and 25 °C was performed as well. No temperature effect was observed compared with 36 °C, thus eliminating the temperature at the intervals investigated as a factor for cubic insulin crystal dissolution, under continuous undersaturation.

Flooding of cubic crystal population with human blood plasma at 36 °C, pH 7.4, flow rate of 30.3 μL/sec, led to an immediate clearing of the recorded cell area, suggesting fast crystal dissolution along with the suggested poor crystal adhesion. In additional experiments at “static” conditions (i.e., without continuous flow across the cell), a reduction of 2.5–5 μm in linear size was observed for 30-μm crystals at a dissolution time of 300 sec. This observation stressed the high dissolution capacity of the natural fluid. In fact, the detached crystals were driven in random directions because of convective currents, and that was practically analogous to flooding at very low flow rates, except for the pure undersaturation conditions when there was directed flow.

Dissolution of Tetragonal Insulin Crystals

The tetragonal insulin crystals had microscopically smooth dissolution in all model flood solutions. In bidistilled water, pH = 5.65, pH = 7.00, 36 °C, a reduction of 2.5–5 μm in linear size was observed for 30-μm crystals at a dissolution time of 300 sec. This observation stressed the high dissolution capacity of the natural fluid. In fact, the detached crystals were driven in random directions because of convective currents, and that was practically analogous to flooding at very low flow rates, except for the pure undersaturation conditions when there was directed flow.

Figure 7. Dissolution of cubic insulin crystals in bidistilled water, pH = 5.65, pH = 7.00, 36 °C. Linear crystal size is 30 μm.
tilded water at pH 5.65 and pH 7.00 at dissolution times as long as 5 h, very slow dissolution rates were detected on a microscale and were nearly independent of the flow rates used (Figure 8). Note that samples left in static conditions at room temperature did not dissolve for a few weeks and stayed surprisingly intact. Thus, the flow rate could have a pronounced effect only on a very large time scale.

In saline solution, tetragonal crystals exhibited considerable dissolution rates (Figure 9) with a recorded dissolution time of 50 min. The dissolution rate was also independent of a particular crystal habit (e.g., hourglass, lemon-like shapes). The same observation held true for all crystal forms investigated in the present study whenever there was diversity in crystal habit.

The first bar in Figure 9 represents a reference study for dissolution rate measurement at pH 7.4. The result implies that small variations in pH (e.g., from subcutaneous tissue toward blood stream) generally have no effect on the dissolution rate.

The use of human blood plasma as flood solution (pH 7.4, 36 °C, flow rate of 45 μL/sec) resulted in significantly higher rates of dissolution, giving about a 5-μm linear size reduction of 30-μm crystals in 90 sec. This confirmed again that the dissolution of insulin crystals has a strong dependence on the particular dissolution environment, especially solution composition.

An advantage of this experimental setup is that every model liquid used as a flood solution was translucent in the Hele–Shaw construction. For the dissolution measurements, it was relied upon that the intrinsic property of protein crystals would adhere to the wall of the crystallization vessel during their nucleation and subsequent crystal growth. Thus, although the flow could carry away loosely attached crystals, a large crystal population still remained for the dissolution rate measurement studies. The exception was the cubic crystals, for which only the rate of crystal detachment was indicative of crystal dissolution rate. Note that the applied method also suggests noninvasive crystal dissolution. Defining the dissolution rate in terms of the reduction of linear crystal size for an exact crystal size and time is reasonable and puts the dissolved amount of insulin on a unified basis. For linear size, the longest crystal dimension was fixed during image analysis. A small drawback of the setup is the limited volume of the reservoir flood solution.

The most pronounced of the effects investigated here for crystal dissolution was the type of flood liquid. This result stressed the importance of the crystal environment with respect to its chemical composition. The dissolution rate of the crystal forms investigated increased in the order: human blood plasma > 0.9% NaCl > bidistilled water, except for the case of monoclinic crystalline insulin. Although it appeared very fragile upon any mother liquor (the solution in which crystals are grown) substitution, clearly discernible disintegration–dissolution patterns can be specified for that particular form. Monoclinic crystalline insulin obtained at similar conditions could be a candidate for rapidly absorbing insulin injectable formulations.

A noticeable dependence of crystal dissolution rate on the crystal form was also observed for any particular flood liquid used; the dependence was more pronounced with composite flood liquids (i.e., saline solution and blood plasma). The flow rate had little impact on the dissolution rate. The effect increased at higher flow rates, and in fact, the flow-rate effect was proportional to the intrinsic crystal propensity for dissolution in any given solution of any insulin crystal form considered. The present dissolution approach at flood conditions was intended to model
an in vivo dynamic environment (mainly with respect to the blood flow). An important fact is that flooded crystals were always in the state of undersaturation. Even the lowest flow rates used here were much higher than insulin molecular diffusion rates during dissolution, and the rate of detachment of insulin molecules or oligomers from the crystal surface (i.e., the surface reaction) was what defined the flow rate effect. Thus, the present dissolution setup avoids some potentially important side effects such as insulin oligomerization during dissolution (16). Note that in the Hele–Shaw construction used, it was hard to precisely define a linear flow rate, and generally it would vary among different positions in the cell. It was measured from floating particles around the crystals, only to confirm that the flow rates used included the range of blood capillary flow rates. According to the goals of this study, representing flow rates as μL/sec (volume of flood liquid flowed through the cell per unit time) was adequate.

The pH of the flood liquid made no considerable contribution to the crystal dissolution rate in the range tested.

**CONCLUSION**

The present investigation constitutes a useful in vitro dissolution model of the in vivo dissolution environment for injectable insulin formulations. Although related to conditions that are more dynamic, the study also presents a reasonable approach to subcutaneous crystalline insulin behavior upon absorption. By presumption, the in vivo process is rather dynamic as well, being restricted neither for insulin diffusion nor by some supersaturation effects during the process of crystal dissolution. Based on this study, different crystalline-based insulin formulations could be further modified with respect to the crystal form and particular liquid phases. Most of the crystal forms investigated here exhibited negligible dissolution in bidistilled water. This could be an important observation for the development of formulations with low dissolution in bidistilled water. This could be an important observation for the development of formulations with low absorption rates. On the other hand, the relatively fast dissolution of fine crystal populations was demonstrated through temperature-dependent dissolution and regrowth.

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