In Situ Study of Insulin Aggregation Induced by Water-Organic Solvent Interface

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Purpose. The aim of this study was to assess insulin stability by monitoring *in situ* time-course of insulin aggregation induced by a water-organic solvent (o/w) interface that occurs during the microencapsulation process.

Methods. Insulin aggregation at a simple o/w interface was monitored spectrophotometrically by detecting the percentage of turbidity changes (%T) at 350 nm. The effects of protein concentration and agitation and the presence of poly (lactic-co-glycolic acid) (PLGA) in methylene chloride (MC) on insulin aggregation were observed. For the 0.72 mg/ml insulin in phosphate-buffered saline (PBS), the effect of nonionic (dodecyl maltoside [DDM]) and anionic (sodium dodecyl sulfate [SDS]) surfactant in PBS were also evaluated at various protein/surfactant mol ratios. The conformation of insulin protected by a 10-fold molar excess of SDS recovered after 1 h of contact with MC was evaluated via circular dichroism (CD) spectroscopy.

Results. A typical turbidity-time profile was represented by a sigmoidal curve. Greater change in %T was observed with increasing insulin concentration, in the presence of PLGA in MC and in the presence of agitation. DDM failed to delay insulin aggregation at all ratios used, whereas a less than 10% change in %T was observed in 1 h when a 10- –~20-fold excess of SDS was used. CD spectra indicated that the presence of insulin in SDS after 1 h of contact with MC qualitatively retained its secondary structure integrity.

Conclusions. An experimental method was designed for an *in situ* assessment of protein stability at the o/w interface.

KEY WORDS: insulin aggregation; protein stability; microsphere preparation; turbidity measurement.

INTRODUCTION

Due to advances in biotechnology, many new protein drugs are currently available. Most of them have to be administered by frequent injections, which hinders their widespread use. Thus, research efforts have been concentrated on sustained-release delivery systems. So far the most popular approach to developing an injectable sustained-release system is to entrap the protein drug into microspheres prepared from biodegradable polymers such as homopolymers or copolymers of lactic and glycolic acids (poly (lactic-co-glycolic acid [PLGA]) (1–4).

These polymers are nontoxic, nonimmunogenic, and thus are well tolerated. A successful delivery system is Lupron Depot delivering the luteinizing hormone-releasing hormone superagonist leuprolide for 3–4 months (2,3) or Nutropin Depot for 1 month of sustained delivery of human growth hormone (4). For water-soluble protein drugs, the water-in-oil (organic phase) (in water double-emulsion), solvent evaporation technique is often employed using a volatile and water-immiscible organic solvent such as methylene chloride (MC), as described elsewhere (1–5).

Two major drawbacks associated with protein microspheres are identified: initial burst effect and incomplete release. The first usually correlates with encapsulation efficiency. To achieve high-loading efficiency and to suppress the initial burst of the drug release, it is required that the viscosity of the inner water phase be increased with additives, and/or by lowering the temperature (3), and/or by decreasing the volume of the aqueous phase containing the drug as compared to organic phase volume of the first emulsion (4). However, making a primary emulsion involves high shear and heat generation to create a large water/organic solvent interfacial area. Proteins can undergo rapid aggregation under this environment, and thus the incomplete release of proteins from PLGA microspheres may be due to aggregation during microsphere fabrication. Efforts have been made to assess protein stability during the preparation process (5,6), within the microsphere after encapsulation (7), or after release (8).

Human insulin was chosen as an unstable, water-soluble model protein for which the physical stability issues have been studied in detail (9–18). The preservation of insulin-native conformation is a condition *sine qua non* for the maintenance of insulin's biological activity for traditional parenteral therapy (17) as well as for novel methods of insulin delivery such as portable continuous insulin pumps (19). It is well accepted that the agitation of aqueous solutions of insulin in the presence of an air-water interface leads to rapid macroscopic aggregation and insulin fibril formation (16). Replacing hydrophobic air-water interface with a Teflon-water interface still leads to efficient aggregation, provided that the insulin solution was agitated (15). Detailed kinetic studies on insulin fibrillation concluded that the reaction could be classified as a nucleation-type phenomenon. Mechanistically, the process is initiated by unfolding of an insulin monomer at the interface (14). It also has been shown that certain surfactants can effectively block aggregation (9,10,15,20).

One can presume that the aggregation process will also occur in the presence of a liquid-liquid hydrophobic interface (i.e., water/immiscible organic solvent [such as water]/dichloromethane), which is present during microsphere fabrication. As compared to a Teflon-water interface, the latter type of interface can be assumed to be continuous. (Both solvent molecules are free to move and can penetrate from one phase to the other.) In other words, the interface is formed by a steep concentration gradients of the solvents involved. Thus, protein molecules dissolved at a high concentration in the aqueous phase can easily reach the interface region with high-organic phase concentration, which favors protein molecule unfolding under supersaturated conditions

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ABBREVIATIONS: CD, circular dichroism; DDM, dodecyl maltoside; MC, methylene chloride; EDTA, ethylenediaminetetraacetic acid; o/w, water-organic solvent; PBS, phosphate-buffered saline; PLGA, poly (lactic-co-glycolic acid); SDS, sodium dodecyl sulfate; %T, percentage of turbidity change.

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(i.e., it provides an optimal environment for the rapid formation of the nucleation centers that are required to trigger macroscopic fibrillation).

Because protein molecules in the inner-water phase may maintain contact with the organic solvent until its evaporation is completed, the main aggregation occurs within a few minutes to 1–~2 h. In this study, insulin aggregation induced by a simple w/o interface was monitored *in situ* by following the percentage of the transmittance change at a wavelength of 350 nm. The designed experimental set-up enabled us to follow and characterize this process kinetically as well as to analyze the effect of individual parameters such as insulin concentration, the presence of polymer (PLGA), and the stabilization effects of nonionic and ionic surfactants.

MATERIALS AND METHODS

Materials

Recombinant human insulin (PENTEX, Zn salt, lot No. 506, 27 IU/mg) was purchased from Beyer Corporation (Kankakee, IL, USA). Ethylenediaminetetraacetic acid (EDTA), dodecyl maltoside (DDM), PLGA (molar ratio of d,l-lactide:glycolide ratio, 50:50; molecular weight, 50,000–75,000) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PLGA was purified via reprecipitation in cold anhydrous ether before use. MC (spectrograde) and sodium dodecyl sulfate (SDS) was purchased from Mallinkrodt Laboratory (Phillipsburg, NJ, USA).

Preparation of Insulin Solution (Zn Removal by EDTA)

Glassware was cleaned with Chromerge (chromium oxide in sulfuric acid) and then was dried in a 120 °C oven before use. For example, the 0.72-mg/ml, Zn-removed insulin solution was prepared by dissolving 4.5 mg of Zn-crystalline insulin powder in 1.0 ml 0.01 M HCl. Then 2.5 ml of a twice-concentrated phosphate-buffered saline (PBS) solution (pH, 7.4; 0.02 M phosphate; 0.29 M NaCl; 0.06% EDTA [w/w; ~1 mM at the final volume]) was added dropwise. The pH of the resultant solution was adjusted to 7.4 by the dropwise addtion of 0.1 N NaOH. The final solution volume was brought to 5.0 ml with the addition of distilled water. The Zn-free insulin solution was filtered through a 0.22-μm Acrodisc low protein-binding syringe filter (Pall Corporation, Ann Arbor, MI, USA). Insulin concentration was determined by UV absorption at 278 nm. The final insulin concentration and its serial dilutions were obtained by adding appropriate amounts of filtered PBS (pH 7.4, 0.01 M phosphate, 0.145 M NaCl, 0.03% EDTA).

To see whether or not the use of EDTA as decribed above leads to effective zinc removal, we compared circular dichroism (CD) spectra (both far and near UV) of a sample to a 'Zn-free' solution (dialyzed against 0.01% NH_4HCO_3/EDTA for 5 days and freeze dried) and the Zn-insulin control. The method described above yielded reproducibly same starting materials.

Turbidity Measurements

Insulin solutions become increasingly turbid as insulin aggregates and forms fibrils (9). Thus, insulin aggregation can be monitored by turbidity changes as detected by a UV/VIS spectrophotometry at 350 nm. Insulin does not absorb at this wavelength.

For turbidity measurement, the apparatus used was a Perkin-Elmer UV/VIS Lambda 19 double-beam spectrophotometer. A temperature-controlled UV quartz cell (1.0 cm pathlength; purchased from Wilmad, Buena, NJ, USA) was used with both sides of the cuvette partially covered to provide a 2.0-mm window through the cell. This allowed limited light passage through the cuvette. MC (with or without PLGA) was placed first (density = 1.325 g/ml) in the bottom of the cuvette, and the insulin solution was then gently placed on top of it to avoid unnecessary shear. Prior to adding insulin solutions over MC, the percentage of turbidity changes (%T) was monitored and the height of the cuvette was adjusted so that we would have the same %T values before the addition of insulin solution, and so that the light beam did not touch the interface. When measuring turbidity, agitation was momentarily stopped. Because the agitation was a mild horizontal orbital motion, it did not create a disruption of the interface. Appropriate blanks were used in all experiments (i.e., the same components except for the protein). The index of refraction change upon agitation was observed to be small (~1 %T unit).

The percentage of transmittance at 350 nm (i.e., the ratio of %T at time $t > 0$ to the %T of the blank) at 25 °C was then recorded as a function of time. Decreased transmittance was associated with increased aggregation at the MC-aqueous solution interface. In the absence of agitation (unstirred condition), the effects of insulin concentration (0.74–2.9 mg/ml) in the presence of MC, PLGA in MC, and SDS in the aqueous phase were studied. To study the shaking condition, a lower concentration range was used (0.38–0.72 mg/ml). Mild shaking (200 rpm) was applied using an orbital shaker (Lab-Line Instruments, Inc., Melrose Park, IL, USA) to ensure the homogeneous dispersion of the aggregates throughout the aqueous phase. %T values were taken every 2 min. A comparison experiment using DDM (a nonionic surfactant) or SDS (an anionic surfactant) to investigate their ability to protect insulin against aggregation was carried out by varying surfactant/protein molar ratios (at a fixed insulin concentration). These surfactants were dissolved after the preparation of insulin solutions in PBS.

CD Spectroscopy

CD spectra were obtained on a Jasco J-720 spectropolarimeter with a 0.1-cm path cuvette for far-UV CD and a 1.0-cm path cuvette for near UV CD on the following samples (see Fig. 6): (1) 'Zn-free' insulin; (2) insulin fully aggregated at o/w interface, which corresponds to the plateau in the turbidity-time profile; (3) insulin/SDS molar ratio of 1:10 before contact with MC; and (4) solution 3 after 1 h of contact with MC. Protein concentrations for CD were 0.5 mg/ml.

RESULTS

Figure 1 shows that CD spectra (both far- and near-UV) of an EDTA-treated sample, of the Zn-free solution (dialyzed). Spectra for both cases overlapped, signifying that insulin in both solutions is in the very similar association state, with significant differences with CD of Zn-insulin control. Although the Zn-insulin control contained 34.8 g atom Zn/

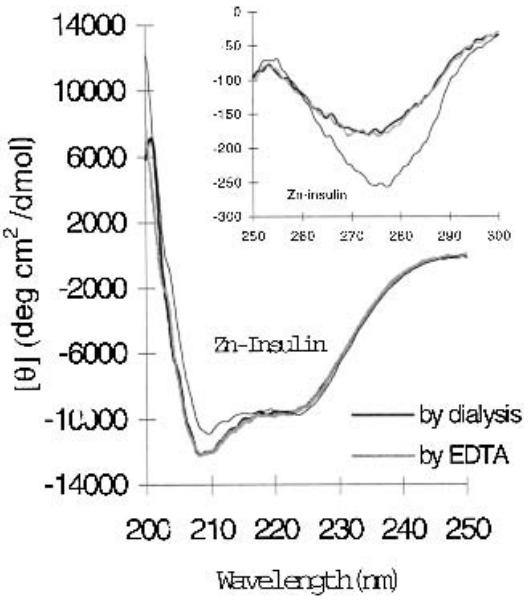

Fig. 1. Far-UV and near-UV (inset) spectra of Zn-free insulin by dialysis, Zn-removed insulin by EDTA, and Zn-insulin control are shown. The insulin concentration = 0.5 mg/ml.

mole of insulin, the Zn-free insulin contained less than 0.05 g atom Zn/mole of insulin, as determined by atomic absorption spectroscopy. Thus, the EDTA-treated sample contains a similar Zn content to the dialyzed sample. Although this is not a strictly zinc-free insulin solution, for our purposes the Zn concentration is low enough to observe the accelerated aggregation of insulin. The EDTA-treated samples were prepared in a reproducible way.

Figure 2 shows the concentration dependence of insulin aggregation for up to 2 h under unstirred conditions. Rapid aggregation was observed at higher insulin concentrations as determined by the decrease in %T at 350 nm vs. time. Without MC, the highest concentrated insulin solution (2.9 mg/ml) did not show a detectable change in %T up to 2 h.

Figure 3a shows that the presence of PLGA further increased the insulin aggregation rate when in contact with MC at a high insulin concentration (2.27 mg/ml). The %T values

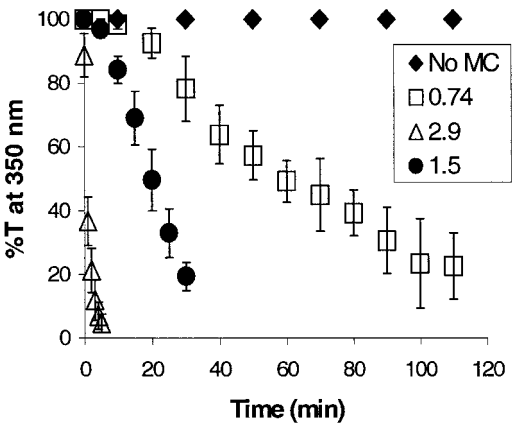

Fig. 2. The effect of insulin concentrations (mg/ml in PBS) on the insulin aggregation rate (unstirred) is shown. The plot of %T at 350 nm vs. time is also shown, as is insulin concentration dependence (in mg/ml in PBS). Without MC, the insulin concentration was 2.9 mg/ml.

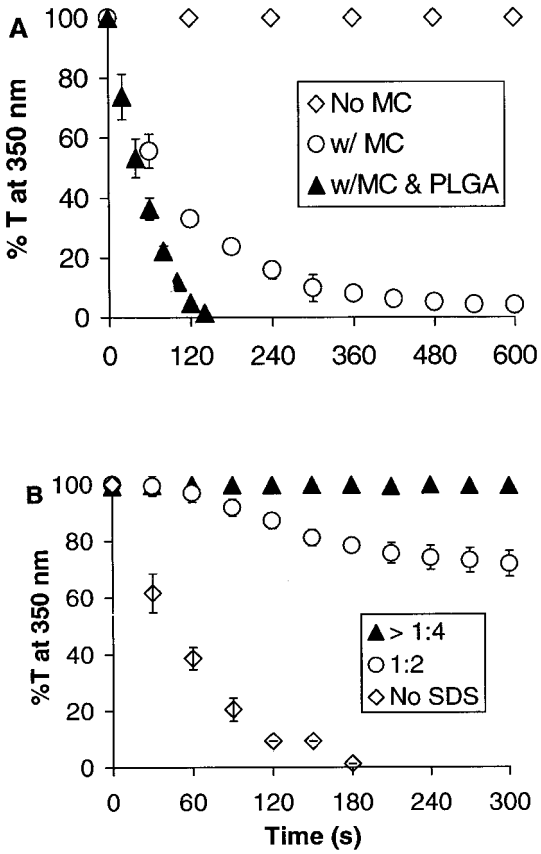

Fig 3. (A) The effect of PLGA in MC (200 mg/ml) on insulin aggregation (unstirred) is shown. Insulin concentration = 2.27 mg/ml. (B) The effect of SDS in PBS on insulin aggregation with different molar ratios of insulin/SDS (unstirred) is shown. Insulin concentration = 2.23 mg/ml. Data points are represented as mean ± SD (n = 3–4).

decreased to 20%T within 3 min in the presence of only MC and within ~1.5 min in the presence of 200 mg/ml PLGA in MC. However, the addition of SDS in PBS decreased the insulin aggregation rate at the PBS-MC interface for insulin at high concentrations (i.e., 2.23 mg/ml), as shown in Fig. 3b. Essentially, no aggregation was observed for 5 min when the SDS concentration was increased to give a molar ratio of 1:4 (insulin/SDS).

Figure 4 presents concentration-dependence curves at a lower insulin concentration range in the presence of mild agitation at 200 rpm. The presence of agitation altered the general time-course for aggregation by an order of magnitude, exhibiting lag time and plateau. At similar concentrations (0.74 mg/ml in Fig. 2 and 0.72 mg/ml in Fig. 4), the transmittance had decreased from 100% to less than 20% within 100 min in the absence of agitation, although in the presence of agitation the same change was accomplished within 10 min.

The effects of DDM and SDS on insulin aggregation and the concentration effects of these additives are shown in Fig. 5, a and b, respectively. The insulin/surfactant molar ratio was varied up to 1:200 for DDM and 1:20 for SDS. Increasing molar excess of DDM slightly decreased insulin aggregation rates at low insulin concentrations (0.72 mg/ml) as the insulin/DDM ratio increased from 1:0, to 1:4, to 1:50, to 1:100. However, a 200-fold molar excess of DDM over insulin showed no stabilization at all. In contrast, the effect of SDS on the de-

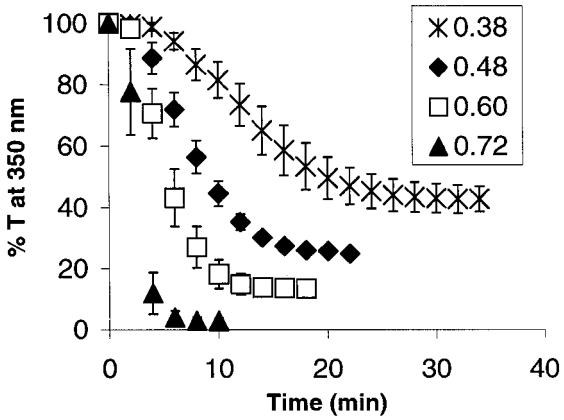

Fig. 4. The effect of insulin concentrations (mg/ml in PBS) on insulin aggregation rate (orbital shaking at 200 rpm) is shown. Data points are represented as mean ± SD (n = 3).

crease of the insulin aggregation rate was more pronounced. The insulin/SDS molar ratios of 1:10 and 1:20 resulted in little change in %T values.

The effects of MC and SDS on insulin's conformation were studied by both far-UV and near-UV CD spectroscopy. The spectra of Zn-free insulin in PBS (0.5 mg/ml), aggregated insulin with no treatment, and Zn-removed insulin containing SDS at a 1:10 molar ratio before and after contact with MC are shown in Fig. 6. Also, we found that the sample with a 1:10 insulin/SDS molar ratio after 1 h of contact with MC at 200

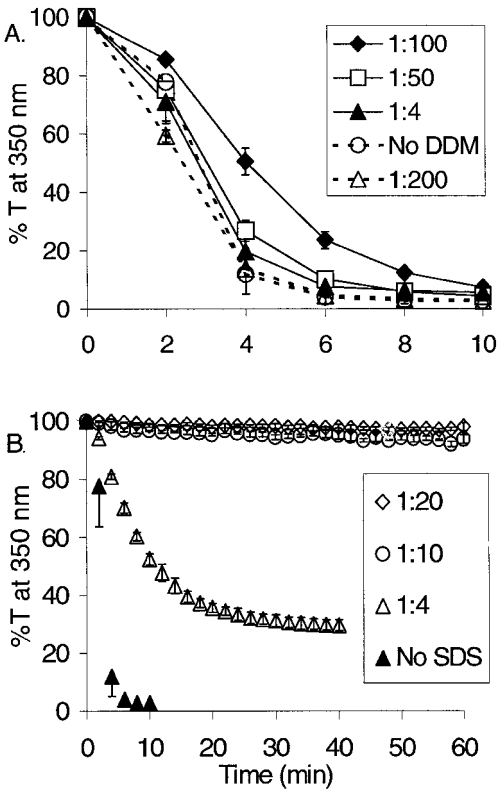

Fig. 5. (A) The effect of DDM in PBS is shown. (B) The effects of SDS in PBS on insulin aggregation profile with varying insulin/surfactant molar ratio (orbital shaking at 200 rpm) is shown. Data points are represented as mean ± SD (n = 3–4). The insulin concentration was 0.72 mg/ml in PBS.

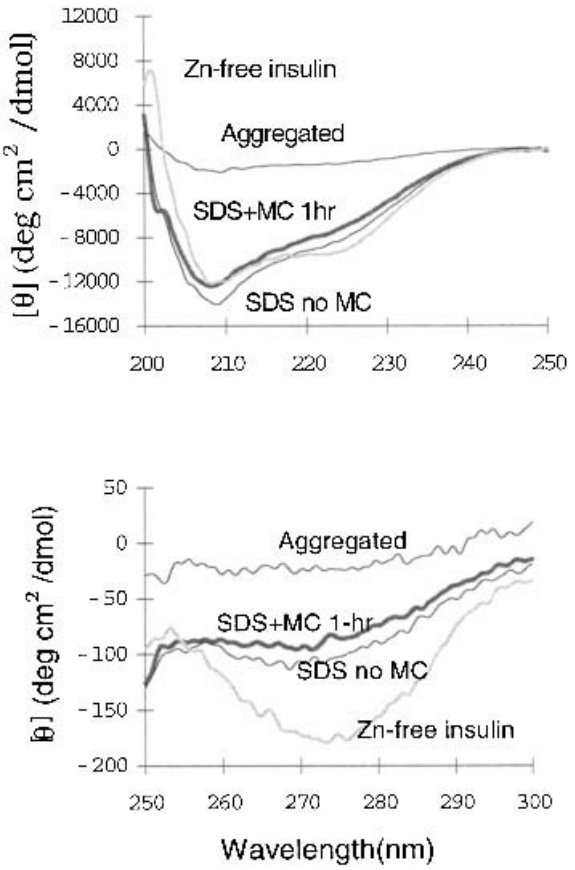

Fig. 6. Far-UV (top) and near-UV (bottom) CD spectra of the following are shown: Zn-free insulin; fully aggregated insulin; insulin/SDS molar ratio of 1:10 before contact with MC; and the same solution after 1 h of contact with MC. The protein concentrations for CD measurements were 0.5 mg/ml.

rpm showed a small decrease in the peak magnitude while maintaining the same qualitative features as the CD of the same solution before contact with MC. This correlates with the decrease in %T at the end of 1 h of contact with MC. In the far-UV CD spectra, the presence of SDS caused a loss of the shoulder at 222 nm and a gain in the peak at 209 nm with respect to Zn-free insulin, as well as the shift at around 200 nm. In the near-UV CD, the attenuation of the negative peak at 276 nm was observed when SDS was present. After the contact with MC for 1 h, a small decrease in CD was observed. Fully aggregated insulin, as a negative control, shows little signals in both far-UV and near-UV CD.

DISCUSSION

Insulin itself was insoluble in the organic solvent, but insulin aggregates formed near the w/o interface as the insulin in PBS was brought into contact with MC, a water-immiscible solvent. Insulin formed insoluble aggregates at the o/w interface as detected and measured by turbidity changes. This was confirmed by the observation that insulin fibril formed at the o/w interface did not redissolve in PBS for months. In a 7 M urea solution, the fibril was dissolved, suggesting that it was a noncovalent aggregate.

It has been reported that the aggregation of insulin exhibits variability from different batches (16). Although the

same batch of insulin was used in our experiments, it should be noted that the use of different batches of insulin could result in major variations in aggregation rates.

In carrying out turbidity experiments, it is interesting to consider how the change in %T due to the aggregation of insulin correlates to the concentration of intact insulin in solution. For instance, for the 1-mg/ml insulin solution at 200 rpm, the %T reaches zero, and at that point the remaining insulin is close to zero. For 0.5 mg/ml, the %T at plateau was ~25% and the percentage of recovered insulin was close to 30% of the initial concentration. Thus, the %T and the concentration of nonaggregated insulin seemed to correlate.

Although it is well established that insulin can undergo aggregation in the presence of air/water or other hydrophobic polymer surfaces with aqueous insulin solutions (9,10,15), the rate of insulin aggregation induced by the MC-water interface (Fig. 4) was an order of magnitude higher. This may be attributed to different characteristics of interfaces. The boundary of the water-MC interface is more mobile than those of the air-water or sold polymer-water interfaces because some solubility of MC in water (2% by weight at room temperature [1]) exists. Hence, organic solvent molecules at the interfacial region can come in direct contact with protein molecules in the aqueous phase, which could facilitate the unfolding process. Insulin, with little or no Zn, in an aqueous solution at a neutral pH may exist as a mixture of monomer, dimer, hexamer, etc. (21). At the interfacial region that lacks hydrophobic interaction due to a high concentration of MC, insulin dimers or higher mers may dissociate easily to result in the production of monomeric species exposed to the interface. Thus, the larger the monomer population at the interface, the faster the formation of the nucleation center (shorter lag time), hence the greater the rate of aggregation as observed in Figs. 2 and 4. This is consistent with the fact that the aggregation rate of monomeric insulin was proportional to insulin concentration (16,18).

As shown in Fig. 4a, the insulin aggregation rate was enhanced in the presence of water-insoluble PLGA in MC. The increase in the aggregation rate in the presence of PLGA can be attributed to a decrease in the o/w interfacial tension as compared to that without PLGA. It was reported that the initial water-MC interfacial tension was 28.4 dynes/cm, whereas it was 21 dynes/cm in the presence of PLGA in MC (22). This is consistent with the observation by Sluzky *et al.* (15) that the rate of insulin aggregation at the air-water interface increased with decreasing air-water interfacial tension.

The two surfactants, SDS (9) and DDM (10,15), have been used previously in stabilizing aqueous insulin formulations. It was shown that DDM was successful in protecting insulin from aggregation at the air-water interfaces as well as at hydrophobic solid-water interfaces under agitated conditions. However, DDM essentially failed to stabilize insulin against aggregation at the MC-water interface in these studies. It is speculated that as the DDM-insulin complexes may not be stable at the MC-rich interfacial region, conformational change may be triggered. Moreover, the excessive amount of DDM seems to be rather destabilizing, as in Fig. 5a. On the contrary, the SDS has significantly reduced insulin aggregation up to 1 h when present in insulin/SDS molar ratios of 1:10 and 1:20. It was suggested that SDS binds with a large number of proteins cooperatively with the negatively charged head groups of SDS pointing outward (23). Also, the net charge of the insulin molecule itself at pH 7.4 is also negative based on pKa values of the acidic/basic groups (17). Therefore, it is possible that the insulin-SDS complex may have a net negative charge. If SDS is adsorbed at the MC/water interface, then charge repulsion between the insulin-SDS complex and the hydrated SDS polar heads at the interface will restrict the insulin-SDS complex from coming in contact with the hydrophobic MC interface.

The effect of SDS on the CD of insulin was previously reported by Shao *et al.*(24). When SDS was used, the far-UV CD showed a loss in the shoulder at 222 nm and a gain at 209 nm, which indicates that the presence of SDS (~1 mM at least) leads to an increase in monomer population. This was manifested in the near-UV CD as the attenuation of peaks at 276 nm, which is suggestive of a different association state. Although the change in CD at around 200 nm was also observed by Shao *et al.* (24), this apparent shift in CD signals with respect to native insulin showed no detrimental effect on insulin bioactivity (24).

We believe that the method described in this article can be applied to other proteins of interest because many exhibit the o/w interface-induced aggregation and turbidity change.

CONCLUSION

This study demonstrates the design of the experiment for the *in situ* monitoring of the time-course of insulin aggregation induced by the o/w interface, which occurs in the process of change of protein entrapment into biodegradable microspheres via the water/o/w double-emulsion method. This method may be used for the routine characterization of protein aggregation at the o/w interface before protein encapsulation. It was found that the use of organic solvent is a destructive source of aggregation of therapeutic proteins, especially at the polymer and solvent interface.

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