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On the stability of insulin delivered through a new glucose-responsive polymeric composite membrane

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Abstract

A new glucose-responsive polymeric composite membrane that provided pulsatile insulin release was developed in our laboratory previously. To develop a clinically useful insulin delivery system, this study was designed to investigate factors influencing insulin stability during delivery by this membrane. The effects of stirring, release duration, insulin concentration and surfactant on insulin stability were studied under both incubation and delivery conditions in a buffer solution at 37°C. The structural change of insulin was characterized by reverse-phase HPLC and circular dichroism. Hydrophobicity of various contact surfaces was determined by contact angle measurement. The results indicated that insulin concentration played an important role in the insulin stability, followed by stirring. Treating the membrane with a non-ionic surfactant prevented insulin denaturation during delivery through the membrane.

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Introduction

Since its first use in man in 1922, insulin delivered via subcutaneous injection has been a major treatment for diabetes. However, this method cannot consistently maintain normal blood glucose levels, due partly to poor patient compliance, resulting in serious complications (Heller 1999; Cryer 2001; Diabetes Report 2003). Therefore, better ways of delivering insulin have been explored, including glucose-responsive insulin delivery systems that can mimic the natural pattern of insulin release in the human body (Brange & Langkjaer 1997; Heller 1997; Trehan & Ali 1998; Cefalu 2001; Kost & Langer 2001; Qiu & Park 2001; Kikuchi & Okano 2002; Zhang & Wu 2002; Belmin & Valensi 2003).

An effective glucose-responsive insulin delivery system should be composed of a glucose-sensing component and an insulin-releasing component. The sensing component detects a change in glucose level and produces a signal that affects the releasing component. The magnitude of the signal increases with increasing glucose concentration, so does the rate of insulin release. Based on this principle, various polymer-based glucose-responsive delivery systems have been designed, most of which are hydrogels that can alter their volume and degree of hydration in response to glucose concentration. These systems can be categorized into direct and indirect glucose-responsive systems (Heller 1997; Wu et al 2003). The direct responsive system utilizes competitive binding of free glucose with a polymer or a protein (e.g. lectin) that forms a complex with another polymer in the absence of free glucose. At a high glucose level, as more free glucose diffuses into the hydrogel, the complexes dissociate and thus the polymer hydrogel swells or reverts to a sol, or the bound insulin derivative (e.g. glycosylated insulin) is displaced, allowing more insulin to diffuse out. The indirect systems make use of conversion of glucose to gluconic acid catalysed by glucose oxidase (GOD), which causes a pH decrease at higher glucose levels. The reduction in pH triggers hydrogel swelling in the case of poly(amines) or shrinking in the case of carboxyl-containing polymers (Heller 1997; Qiu & Park 2001; Zhang & Wu 2002; Wu et al 2003). In the former case, the rate of insulin diffusion in the hydrogel increases, while in the latter, more pores are generated owing to hydrogel collapse leading to faster insulin release.

Four types of indirect glucose sensitive systems have been investigated, namely bulk hydrogel membranes (Ishihara et al 1984; Albin et al 1985), bulk hydrogel matrixes (Traitel et al 2000), grafted porous membranes (Iwata & Matsuda 1988; Ito et al 1989; Cartier et al 1995) and polymeric composite membranes (Zhang & Wu 2002). Bulk matrix or membranes of poly(amines) hydrogels containing entrapped glucose oxidase have been investigated for insulin delivery by Horbett and coworkers and other groups since the 1980s (Ishihara 1984; Albin et al 1985; Kost et al 1985; Traitel et al 2000). Because of the low mechanical strength of bulk membranes and slow response of bulk matrixes, and the low yield of enzyme immobilization in grafted membranes, composite membranes have been devised (Zhang & Wu 2002).

The glucose-responsive polymeric composite membranes were prepared by physical incorporation of pH-responsive poly(*N*-isopropylacrylamide-co-methacrylic acid) nanoparticles, GOD and catalase (for improved GOD efficiency (Jung et al 2000; Podual et al 2000)) in a matrix of a hydrophobic polymer. As the glucose level increases from a base line, more glucose diffuses into the membrane and reacts with oxygen and water, as catalysed by GOD, producing more gluconic acid. When the rate of gluconic acid production is greater than its rate of diffusion out of the membrane, it accumulates in the membrane, causing a pH drop in the membrane. Consequently, the nanoparticles shrink leaving larger pores in the membrane thus allowing more insulin to diffuse through. The increase in the rate of insulin permeation through such a membrane reached more than 8 fold as the glucose concentration was raised from 50 mg dL^{-1} to 400 mg dL^{-1} (Zhang & Wu 2002).

Unlike the grafted membrane, no chemical reaction is required for immobilization of the enzymes. Thus, much higher yield and activity of the enzymes were obtained (Zhang & Wu 2002). Owing to the use of the nano-sized particles that experience rapid shrinking or swelling, the change in insulin permeability could be detected within 5–15 min, which is very fast compared with other systems. The composite structure also imparts better mechanical strength to the membrane than hydrogel alone (Yam et al 2000; Zhang & Wu 2002; Wu & Yam 2003). Furthermore, our preliminary study suggested good biocompatibility of the membrane system due to the presence of the hydrogel nanoparticles. Encouraged by these promising results, we intend to pursue further investigations on the therapeutic effect of delivered insulin and to develop a stable insulin formulation for in-vivo application. Before embarking on in-vivo studies, it is necessary to investigate the stability of insulin delivered by the membrane since insulin may undergo chemical decomposition and physical denaturation, such as aggregation, resulting in conformational changes of insulin and lower therapeutic efficacy (Manning et al 1989; Brange 1994).

Aggregation of insulin in aqueous solutions has often been encountered when it is delivered through an infusion pump or from a polymeric device over a long time (Creque et al 1980; Irsigler & Kritz 1980; Lougheed et al 1980; James et al 1981; Brange & Havelund 1983a, b; Brennan et al 1985; Brown et al 1986). The aggregation not only drastically reduces the biological activity of insulin, but also blocks delivery routes, which causes a serious problem to drug delivery systems (Lougheed et al 1980; James et al 1981). It has been proposed that insulin adsorption onto hydrophobic surfaces is the key step to insulin aggregation (Sluzky et al 1991, 1992; Nielsen et al 2001), which may be prevented by using surfactants in insulin solutions (Brange et al 1997). To deliver insulin for a long time, the original conformation of insulin must be maintained throughout the whole delivery period. Therefore, the primary objective of this work was to study the factors influencing insulin stability during its delivery by the glucose-responsive composite membrane. The effects of various contact surfaces, delivery conditions and insulin concentration on the insulin stability were investigated, and the use of non-ionic surfactants to stabilize insulin during delivery was explored.

Materials and Methods

Materials

Methacrylic acid (Aldrich) was made inhibitor free by distillation. *N*-isopropylacrylamide (Eastman Kodak) was purified by recrystallization from hexane and toluene. *N,N′*-methylenebisacrylamide (Aldrich), sodium dodecyl sulfate (SDS; Mallinckrodt), potassium persulfate (Aldrich) and ethylcellulose (premium, viscosity 100; a gift from Dow Chemicals) were used as received. Insulin (from porcine pancreas), glucose oxidase (GOD, type X-S, from *Aspergillus niger*) and catalase (from *A. niger*) were purchased from Sigma Co. and dextrose (anhydrous D-glucose) was obtained from Fisher. Non-ionic sugar-based surfactants n-octyl-β-D-glucopyranoside (NOGP, MW 292.4) was purchased from Sigma, and D-α-tocoheryl polyethylene glycol 1000 succinate (vitamin E TPGS, MW 1513), a vitamin E derivative, was a gift from Eastman Chemical Canada, Inc. The chemical structures of the surfactants are shown in Figure 1. All these chemicals were used without further purification unless otherwise specified.

Synthesis of poly(*N*-isopropylacrylamide/methacrylic acid) nanoparticles

Nanoparticles of poly(*N*-isopropylacrylamide/methacrylic acid) were synthesized by an aqueous dispersion polymerization process (Wu & Lee 1993). *N*-isopropylacrylamide, methacrylic acid and *N,N′*-methylenebisacrylamide, at a mole ratio 1:1:0.068, were dissolved in de-ionized distilled (DDI) water to give a total concentration of 135 mM. A small amount of SDS (0.4 mM) was used to stabilize the resultant nanoparticles. The solution was heated to 70 °C and purged with N_2, followed by addition of 2.1 mM potassium persulfate to initiate the polymerization. The reaction was carried out at 70 °C under an N_2 blanket for 4 h with constant stirring at 200 rev min^{-1}. The nanoparticles were then purified by membrane dialysis against DDI water, using a dialysis tubing (Spectra/Pro; Fisher

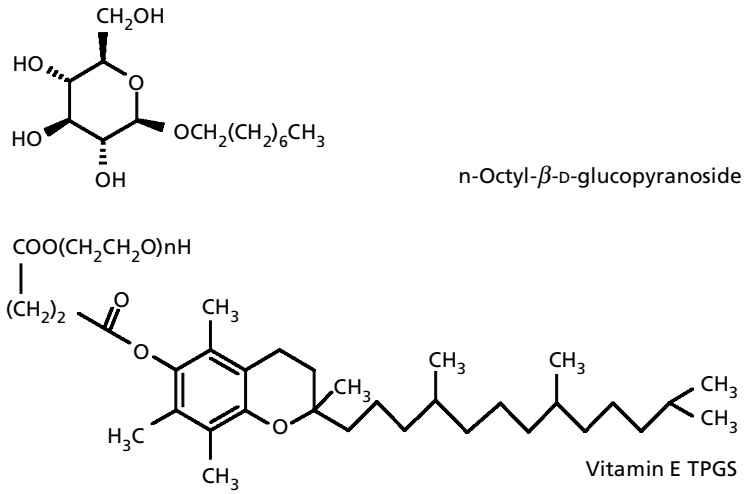

Figure 1 Chemical structures of the two surfactants used.

Scientific) of a molecular weight cut-off of 12 000–14 000. The particle size and size distribution was determined by a dynamic laser scattering particle sizer (NICOMP, Model 370). The nanoparticles were about 500 nm in diameter in water at room temperature.

Preparation of glucose-responsive membranes

A solution casting method was employed to prepare the membranes (Yam & Wu 1999, 2000; Zhang & Wu 2002). The purified nanoparticles were first dried and dispersed in 100% ethanol together with both GOD and catalase. Ethylcellulose dissolved in 100% ethanol was then added to this mixture and stirred manually until uniformity was reached. The amount of nanoparticles, ethylcellulose and enzymes was adjusted according to the needs. In a typical preparation, 0.15 g of dried poly(*N*-isopropylacrylamide/methacrylic acid) nanoparticles, 5 mg of GOD and 1.44 mg of catalase were mixed in 20 g of ethylcellulose ethanol solution. The mixture was poured into a glass dish and kept in a desiccator. After evaporation of the solvent, a membrane of ~0.1 mm in thickness and 6 cm in diameter was obtained, consisting of 35% (w/w) of poly(*N*-isopropylacrylamide/methacrylic acid) nanoparticles with 1:1 molar ratio of *N*-isopropylacrylamide to methacrylic acid, 1% (w/w) of GOD and 3.5% (w/w) of catalase. The actual thickness of wet membranes was measured with a micrometer (Fowler Inc.). The membrane was cleaned with DDI water and stored in a pH 7.4 phosphate buffer solution at 4 °C for future use.

Determination of insulin stability

The stability of insulin was studied under either delivery (see Delivery study) or incubation (see Incubation study) conditions. All experiments were performed using side-by-side diffusion cells between which a piece of the membrane was mounted. The two cells, each with a volume of 3 mL and an exposure area for permeation of 0.63 cm^2, were linked with a water bath (Haake D8) that maintained the temperature at 37 °C. A stock solution of insulin was prepared by dissolving insulin powder in a minimal volume of 0.1 M HCl, and then diluted with pH 7.4 phosphate-buffered saline (PBS) (10 mM, 0.15 M NaCl) to a concentration of 5 mg mL^{-1}. The final pH of the stock solution was adjusted to 7.4 using 0.1 M NaOH. Insulin solutions of various concentrations (5, 10, 50, 5000 $\mu g\,mL^{-1}$) were prepared by dilution of the stock solution using pH 7.4 PBS. After filling with a solution, the cells were sealed with Parafilm, leaving about 0.6 mL air space above the solution. Reversed-phase high-performance liquid chromatography (RP-HPLC) and circular dichroism (CD) were employed to determine structural/conformational changes of insulin.

Delivery study

To mimic the delivery process, one cell (donor) was filled with the 5 mg mL^{-1} insulin solution, while the other cell (receptor) was filled with pH 7.4 PBS (10 mM, 0.15 M NaCl) and 200 mg dL^{-1} of glucose as a releasing medium. The solutions were kept at 37 °C, unstirred or stirred by a magnetic stirring bar (Star Head, 8 mm × 6 mm, coated with Teflon; Fisher) at about 800 rev min^{-1}. Samples were taken from both cells and assayed using RP-HPLC at a time interval of 1 h and examined by CD.

Incubation study

The experiments were carried out using a similar method as described above, except that both cells were filled with the same insulin solution with a concentration of 5, 10, 50 or 5000 $\mu g\,mL^{-1}$. The solution was stirred by a magnetic stirring bar or was not stirred. This study was designed to differentiate the effect of insulin permeation through the membrane on the insulin stability from other factors, such as insulin concentration and contact time.

Insulin stability in the presence of a surfactant

In the delivery study, the membrane was first soaked in a surfactant-containing releasing medium overnight at 4 °C before being mounted onto the cells or pretreated with the medium for 30 min. Then the donor cell was filled with the insulin stock solution, while the receptor cell was filled with a medium containing a surfactant of a concentration equivalent to half of its CMC (critical micellar concentration) — 10 mM for NOGP and 67 μM for vitamin E TPGS. In the incubation study, both cells were filled with the surfactant-containing releasing medium, and equilibrated at 37 °C for about 30 min to treat the membrane surface. Then the release medium was replaced by a surfactant-containing insulin solution. The solutions were stirred with magnetic stirring bars at about 800 rev min^{-1}.

RP-HPLC

A Waters HPLC system equipped with a solvent delivery system (an automated gradient controller, model 680), two HPLC pumps, (model 510 & 501), a UV detector (model 481) and a C_{18} column (5 μm, 250 mm × 4.6 mm i.d.; Sigma, USA) was used to examine insulin aggregation. The column was equilibrated, before injection of samples, with 80% of solution A (aqueous solution–acetonitrile, 80:20 v/v) and 20% solution B (aqueous solution–acetonitrile, 50:50 v/v) at a flow rate of 1 mL min^{-1}. The aqueous solution was prepared by dissolving 28.4 g of sodium sulfate and 2.7 mL of phosphoric acid in 1000 mL of DDI water and the pH of the solution was adjusted to 2.3 using ethanolamine. Insulin solution (20 μL) was injected and eluted with a gradient starting at 80% solution A and 20% solution B and ending at 50% solution A and 50% solution B over 20 min. The absorbance of the eluant was recorded at 214 nm, and the area under the curve was measured for calculation of insulin concentration based on the calibration curves.

Circular dichroism (CD)

CD spectra of insulin were recorded at 37 °C on an AVIV model 62A DS CD instrument (AVIV associates, Lakewood, NJ). The spectra of insulin samples with concentrations about 0.1 mg mL^{-1} were compared with that of fresh insulin. The CD data were expressed as the mean residue ellipticity that was measured at 300–200 nm.

Contact angle measurement

To evaluate the hydrophobicity of various surfaces in contact with insulin and their possible effects on insulin stability, a goniometer (Model 100-00; Rame-Hart Inc.) was employed to measure contact angles of glycerol on the diffusion cell, the stirring bar and dry membranes with 0% or 35% of the nanoparticles at ambient temperature. Each of the samples was placed in an environment-controlled chamber and equilibrated for 5 min before the measurement. Five measurements were undertaken and the average ± standard deviation of five readings was reported.

Statistical analysis

All the experiments described above were conducted at least three times. Except for the raw data of original plots, all numerical values determined were input into spreadsheets of Microsoft Excel and analysed using the built-in statistical package. The average of the repeats and standard deviation of the data were reported. For comparison of the effect of insulin concentration and surfactants on insulin stability, the rates of insulin aggregation were obtained from the curves of fraction of insulin unchanged vs time and analysed using two-tailed t-test with 95% confidence interval. The difference in the aggregation rates under various testing conditions with a t value deviating from critical t statistic ($t_{0.05,\text{two-tailed,df}}$) was considered statistically significant (Jones 2002).

Results

HPLC chromatographs

A shift of the retention time of insulin is an indication of insulin aggregation. The insulin from a freshly prepared solution had a retention time of 9 min (Figure 2A), as did the insulin in the donor cell for up to 3 days in a delivery experiment, even without a surfactant (Figure 2B). However, the retention time for insulin released into the receptor cell shifted to 3 min after 1 h (Figure 2C, D). The area of the peak at 3 min also increased with time due to more insulin molecules that permeated and thereafter denatured.

Figure 3 presents the chromatographs of insulin at various times in an incubation study using 50 μg mL^{-1} insulin solution without a surfactant (Figure 3A–D) and with 10 mM NOGP at 24 h (Figure 3E). The peak shifted to 3 min after 24 h in the absence of the surfactant but remained unchanged in the presence of the surfactant. The peak at 3 min only showed up after the peak at 9 min disappeared for a while (data not shown), indicating a lag time between the transformation of the insulin forms. Besides, the area for the peak at 9 min decreased, while the area for peak at 3 min increased, with time.

CD spectra of insulin

Figure 4 portrays the CD spectra of 0.1 mg mL^{-1} insulin solution with no surfactant after 3 days in a delivery study with stirring. The helical native structure of insulin was present in the sample from the donor cell (Figure 4A), while this conformation was lost in the sample from the receptor cell (Figure 4B). The latter was, in fact, a characteristic spectrum of a random coil structure, indicating denaturation of insulin (Hovgaard et al 1996; Kwon et al 2001). This result is consistent with the result from the HPLC assay. As the insulin concentration in the receptor cell was too low to be detected by CD at early times, no

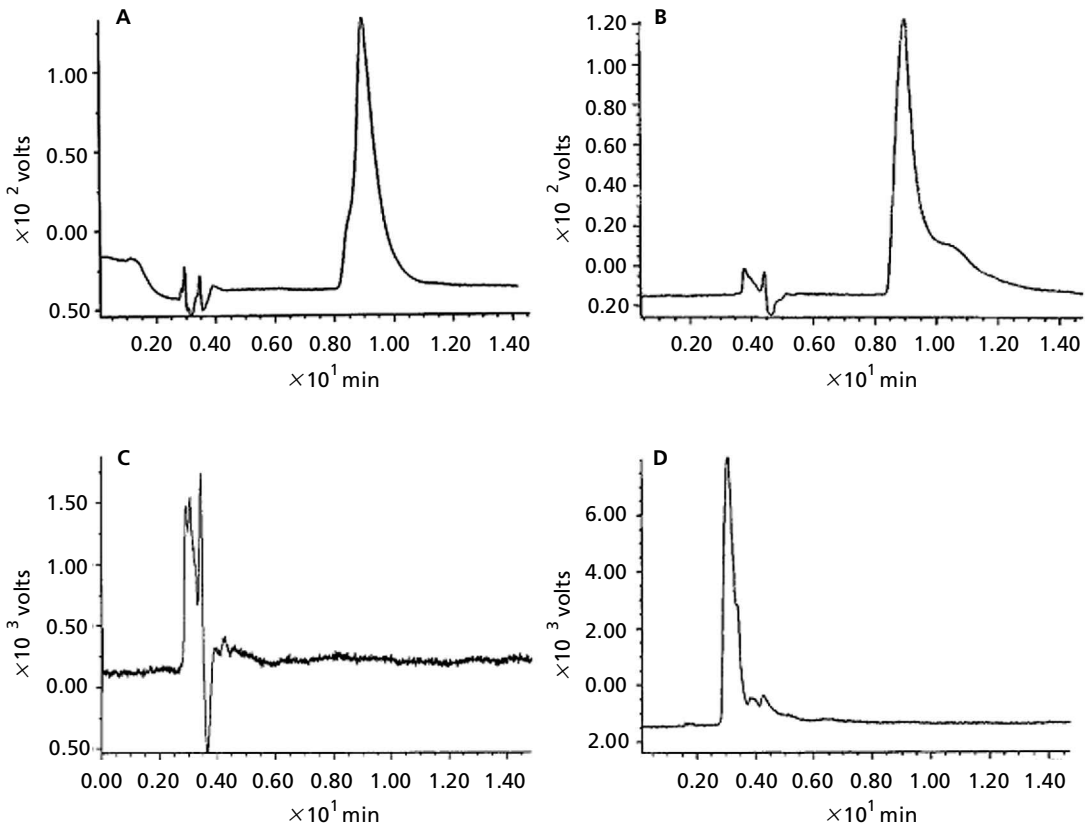

Figure 2 HPLC chromatographs for insulin samples taken from the donor cell at 0 h (A) and 72 h (B), or the receptor cell at 1 h (C) and 10 h (D), without a surfactant under the delivery condition at 37 °C with stirring. Donor cell samples were diluted from the solution in the donor cell to a concentration of $\sim 100\,\mu g\,mL^{-1}$. The measurements were run in triplicate with good reproducibility though only graphs of one experiment are presented.

CD spectrum was acquired before 3 days of the delivery test.

Stirring effect

Experiments were conducted under the delivery or the incubation settings with or without stirring of the solution or medium. In the delivery study, no obvious stirring effect was observed. The results of HPLC and CD studies, with or without stirring, all showed that the insulin in the donor cell remained unchanged up to 3 days, while the insulin released into the receptor cell was only detected as a changed form. However, in the incubation experiments, stirring shortened the insulin stability. The insulin in a solution with a concentration as low as $5\,\mu g\,mL^{-1}$ remained intact for 8 h without stirring, whereas all native form of insulin was gone after 6 h with stirring, as indicated by disappearance of the 9-min peak.

Effect of insulin concentration

As described above, insulin in the donor cell, with a concentration of $5\,mg\,mL^{-1}$, remained unchanged for 3 days, while insulin in the receptor cell denatured as early as it could be measured, regardless of agitation condition. This result suggests that insulin concentration may play a more important role than stirring in the insulin denaturation. To further investigate the effect of insulin concentration, incubating solutions of various concentrations in both cells was conducted and the samples were analysed by RP-HPLC. The area under the 9-min peak relative to that for freshly prepared insulin (i.e., fraction of insulin unchanged) was used as a measure of insulin stability.

Figure 5 shows a plot of fraction of insulin unchanged against time for different insulin concentrations in the absence of a surfactant (n = 3). Apparently, the native insulin diminished with time and the diminishing rate increased as insulin concentration decreased. The time for the 9-min peak to completely disappear decreased from 24 h to 16 h to 6 h as insulin concentration was reduced from $50\,\mu g\,mL^{-1}$ to $10\,\mu g\,mL^{-1}$ to $5\,\mu g\,mL^{-1}$. In contrast, there was no detectable change in the area of the 9-min peak for $5000\,\mu g\,mL^{-1}$ ($5\,mg\,mL^{-1}$) insulin solution for up to 24 h, indicating that about all the insulin maintains its stability in the $5000\,\mu g\,mL^{-1}$ solution for at least one day. The rates of insulin aggregation, evaluated from the curves in Figure 5 using linear regression as first approximation, for the four concentrations were -0.1660 ± 0.0054 ($5\,\mu g\,mL^{-1}$), -0.0634 ± 0.00026 ($10\,\mu g\,mL^{-1}$), -0.0443 ± 0.00124 ($50\,\mu g\,mL^{-1}$) and -0.00019 ± 0.00021 ($5000\,\mu g\,mL^{-1}$).

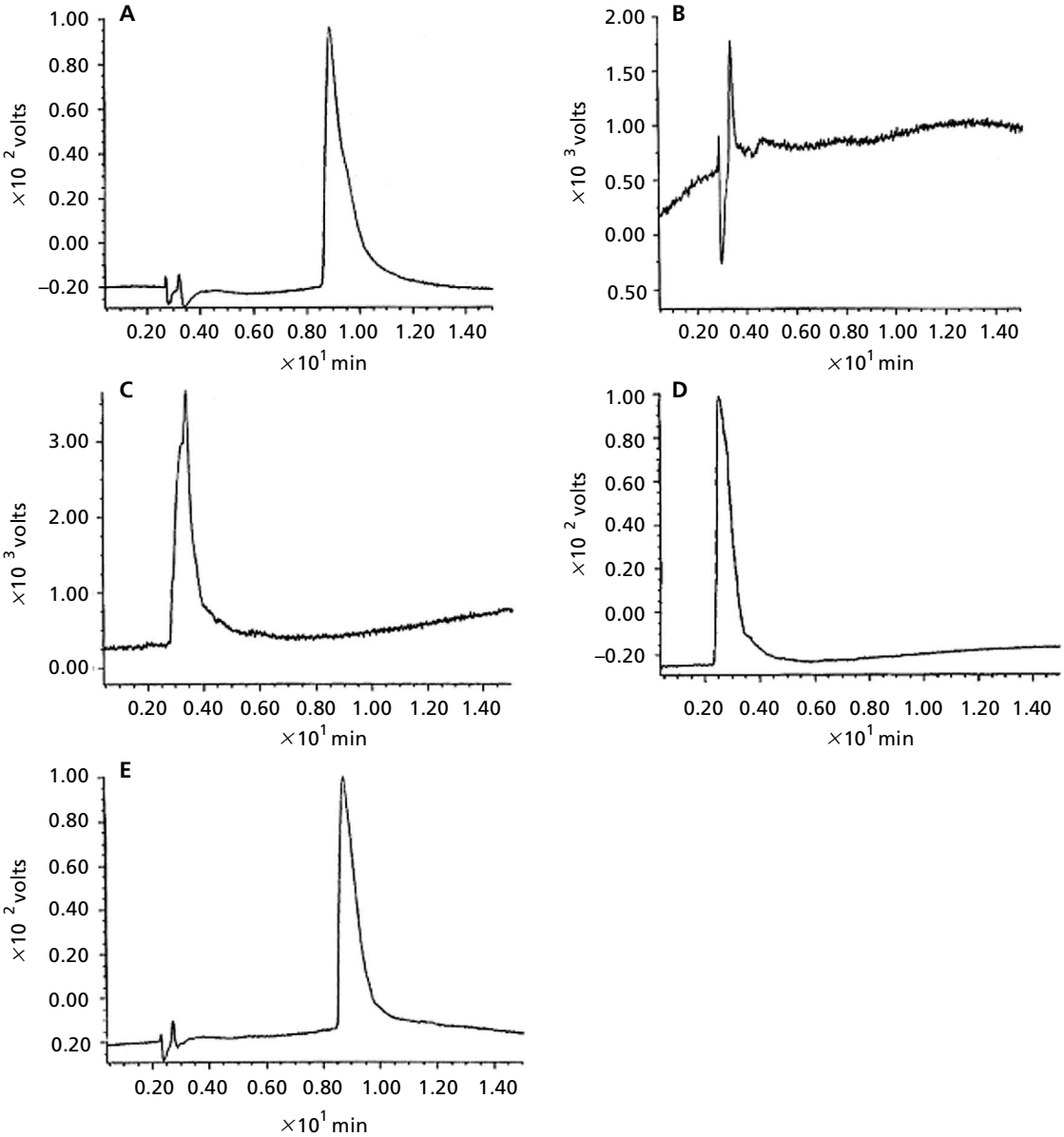

Figure 3 HPLC chromatographs of samples taken from a solution of 50 μg mL^{-1} insulin at 0 h (A), 24 h (B), 48 h (C) and 72 h (D), under the incubation condition at 37 °C with stirring, without a surfactant or at 24 h (E) in the presence of 10 mM n-octyl-β-D-glucopyranoside. The measurements were run in triplicate with good reproducibility though only graphs of one experiment are presented.

The t values of the differences ranged from 10.7 to 68.2, all beyond the critical t statistic ($t_{0.05,\text{two-tailed,df}} = 4.303$–$7.65$ for subpopulations with different variances) for acceptance of the hypothesis that all rates are equal. This result indicates that the effect of insulin concentration on insulin stability was statistically significant.

Effect of surfactants

Two non-ionic surfactants, NOGP and vitamin E TPGS, were used at 10 mM or 67 μM, respectively, to study their effect on insulin stability. The experiment was initially performed under the incubation condition. Figure 6 depicts the fraction of unchanged insulin versus time for a 10 μg mL^{-1} solution with or without a surfactant. All insulin changed after 16 h in the absence of a surfactant, whereas after 24 h about 20% of insulin remained in a solution with vitamin E TPGS, and almost all the insulin maintained its original form in the presence of NOGP. The rates of insulin aggregation were evaluated to be -0.06344 ± 0.00026 (without surfactant), -0.02739 ± 0.00689 (with vitamin E TPGS) and -0.00039 ± 0.00065 (with NOGP). The t values of differences between these rates (6.84, 5.15, 6.84) were beyond the critical t statistic

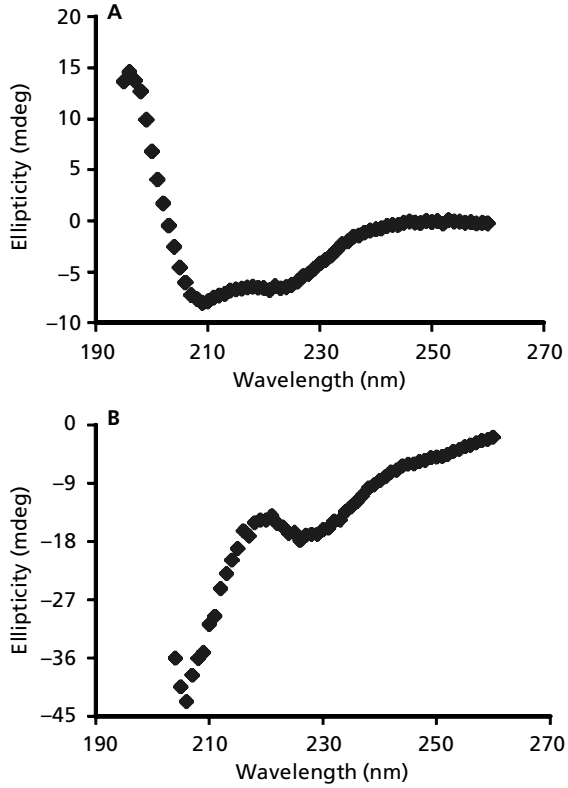

Figure 4 CD spectra of insulin samples from the donor cell (A) or the receptor cell (B) without a surfactant at day 3 under the delivery condition at 37 °C with stirring. The same results were obtained with no stirring. This method has an error $<1\%$ in repeated measurements at insulin concentrations $\geq 100\,\mu g\,mL^{-1}$.

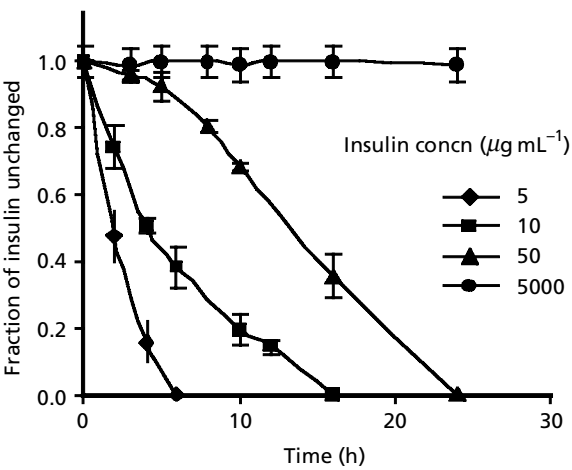

Figure 5 Fraction of insulin unchanged in solutions of varied insulin concentrations without a surfactant under the incubation condition at 37 °C with stirring. The data are reported as average ± s.d., n = 3.

($t_{0.05,\text{two-tailed,df}} = 4.303$), leading to a conclusion of statistical significance of the difference. Apparently, the insulin stability was dramatically improved by the use of the

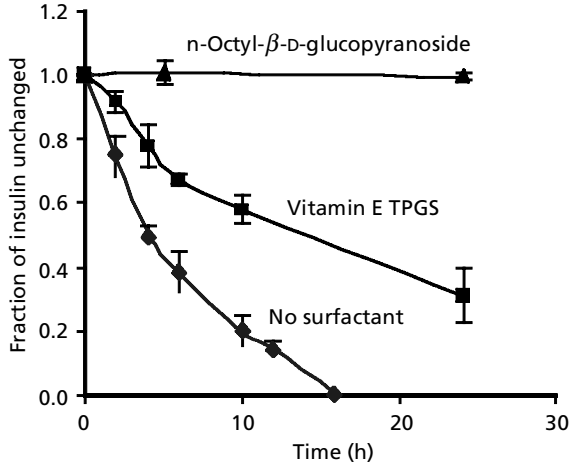

Figure 6 Fraction of insulin unchanged in $10\,\mu g\,mL^{-1}$ insulin in the presence of a surfactant at its concentration of half-CMC compared with that without a surfactant under the incubation condition at 37 °C with stirring. The data are reported as average ± s.d., n = 3.

surfactants, especially NOGP. As presented previously, no HPLC peak for denatured insulin (eluted at 3 min) was detected for the $50\,\mu g\,mL^{-1}$ insulin solution with 10 mM NOGP after 24 h (Figure 3E). Encouragingly, the use of 10 mM NOGP can prevent insulin from denaturing for at least 24 h even if the concentration is as low as $5\,\mu g\,mL^{-1}$. Without a surfactant, all insulin in a $5\,\mu g\,mL^{-1}$ solution denatured after 6 h, as depicted in Figure 5. Virtually identical curves of unchanged insulin versus time were evident in the presence of NOGP (data not shown) for the three concentrations tested (5, 10 and $50\,\mu g\,mL^{-1}$), which suggests that the concentration-dependent insulin denaturation was suppressed by the surfactant.

Based on the result of the incubation study, delivery experiments were performed with addition of 10 mM NOGP in the receptor cell. When the membrane was soaked in the surfactant-containing releasing medium overnight before the experiment, the samples from the receptor cell had a retention time of 9 min in the HPLC chromatograph. In contrast, when the membrane was treated with the medium for 30 min, only a peak at 3 min was detected. This result indicates that the way of treating the membrane with the surfactant is critical in the stabilization of insulin.

Contact angles

The contact angles of glycerol on various surfaces were measured in quintuplicate and are reported as average ± standard deviation as follows: $36.3 \pm 0.3°$ for the diffusion cell (glass surface), $46.0 \pm 0.7°$ for the dry membrane with 35% of nanoparticles, $64.6 \pm 2.1°$ for the dry membrane of ethylcellulose without the particles and $86.3 \pm 1.2°$ for the stirring bar (Teflon surface). In other words, the stirring bar, with the highest hydrophobicity, had the highest potential, while the diffusion cell had the lowest potential, to induce insulin denaturation. Obviously the

presence of nanoparticle reduces the hydrophobicity of the membrane significantly.

Discussion

Effects of insulin concentration

As demonstrated in the delivery study, insulin in the donor cell was more stable than insulin released into the receptor cell no matter whether the solution was stirred or not, which is believed to be a consequence of the difference in insulin concentration. The receptor cell initially contained no insulin, and gradually received up to $\sim 100\ \mu g\ mL^{-1}$ insulin after 3 days. However, the concentration of insulin in the donor cell was $5\ mg\ mL^{-1}$ in the beginning and decreased slightly during the delivery study — much higher than that in the receptor cell. The result from the incubation study using identical solutions of constant concentrations in both diffusion cells has confirmed that insulin concentration is a key factor influencing insulin stability.

The effect of concentration on insulin stability may be explained by the concentration dependence of the monomeric form of insulin. In a solution, insulin monomer coexists with dimer and hexamer and its proportion increases with a decrease in total concentration of insulin. It has been suggested that insulin monomer is essential to insulin aggregation, which occurs by two steps: first, insulin monomer undergoes partial unfolding and then the partially denatured monomer molecules combine with one another to form aggregates (Sluzky et al 1991, 1992; Nielsen et al 2001). At lower concentrations, more monomeric form is present and thus more aggregates can be produced. Therefore, insulin stability decreases with decreasing insulin concentration in aqueous solutions of a neutral pH (Jeffrey et al 1976; Creque et al 1980; Brange & Havelund 1983a, b; Dathe et al 1990).

It appears, in this study, that a concentration of $5\ mg\ mL^{-1}$ is sufficient for maintaining insulin stability in pH 7.4 PBS at 37 °C with or without stirring. Since this concentration can be achieved inside a delivery system, the challenge is how to prevent insulin from denaturing during the process of diffusion through the membrane and release into the medium.

Effect of hydrophobic surface and agitation

In addition to insulin concentration, hydrophobic surface and agitation strongly influence insulin stability. Some studies have demonstrated that both agitation and hydrophobic surface can trigger insulin aggregation in neutral insulin solutions at moderate temperatures (Sluzky et al 1991, 1992). Previous investigations on insulin pumps or insulin delivery systems have revealed that insulin has a strong tendency to aggregate at hydrophobic surfaces and its stability decreases with increasing surface hydrophobicity and contact surface area (Creque et al 1980; Irsigler & Kritz 1980; Lougheed et al 1980; James et al 1981; Brange & Havelund 1983a, b; Brennan et al 1985; Brown et al 1986). In this study, the involved surfaces included glass (diffusion cells), Teflon (stirring bars), polymer membranes and air–water interface. Glass is relatively inert to insulin due to its high hydrophilicity (Sluzky et al 1991, 1992). The composite membrane is much less hydrophobic than ethylcellulose, as shown by the contact angles, because of the presence of 35% (w/w) of hydrogel nanoparticles of poly(*N*-isopropylacrylamide/methacrylic acid) that has a similar composition to the hydrogel beads investigated by Ramkissoon-Ganorkar et al (1999), through which permeated insulin maintained its original conformation. Hence, we believe that interaction between insulin and the hydrophobic domains of the membrane, instead of the nanoparticles, is responsible for the insulin aggregation during delivery.

The use of the stirring bar introduces a very hydrophobic surface of Teflon to the system, which can effectively unfold insulin (Sluzky et al 1991, 1992). On the other hand, agitation at $800\ rev\ min^{-1}$ produces a quickly renewed hydrophobic air–water interface and increases the rate of insulin transport to the air–water interface and other hydrophobic surfaces. The formation of insulin aggregates is a slow process, as reported in literature (Brange 1994; Brange et al 1997) and reflected by a time lag between the disappearance of 9-min peak and the appearance of the 3-min peak observed in this work. Hence an action like the agitation that accelerates mass transport of insulin would speed insulin aggregation. The effect of agitation seems to be lessened at higher insulin concentrations. Even if being stirred for 3 days, the native insulin in the donor cell remains, suggesting that the concentration effect could overcome the agitation effect and the presence of insulin monomer is a critical factor in insulin aggregation.

Effect of surfactant and membrane treatment

This work has revealed that non-ionic surfactants, NOGP and vitamin E TPGS, can preserve insulin stability during delivery, which is consistent with the finding that non-ionic surfactants can prevent insulin from aggregating in aqueous media (Sefton & Antonacci 1983). The protective effect of the surfactants probably stems from their ability to cover hydrophobic surfaces that would trigger insulin aggregation. The higher effectiveness of NOGP than vitamin E TPGS in improving insulin stability is likely due to its smaller molecular size (MW 292.4 compared with MW 1513 for vitamin E TPGS). Smaller molecules can penetrate both large and small pores and cover more hydrophobic surface than large ones (Sluzky et al 1992), thus exhibiting a greater effect.

Interestingly the protective effect of surfactant depends upon the experimental condition and the way by which the membrane is treated with a surfactant. In the incubation experiment, the insulin remains stable for at least 24 h at a concentration as low as $5\ \mu g\ mL^{-1}$, even if the membrane is treated with a surfactant solution only for 30 min. But in the delivery experiment, when the membrane is treated for 30 min, only a changed form of insulin is detected from the donor cell; only when the membrane is soaked in the

surfactant solution overnight does the permeated insulin keep it original form for at least 6 h. This observation suggests that contact of insulin with the internal channels of the membrane during permeation somehow causes insulin denaturation.

In the incubation experiments, the same insulin solution is placed in both donor and receptor cells. Hence there is no net flow of insulin from one side to the other, which minimizes the contact of insulin with the inner surface of the membrane. In other words, the main contact of insulin with the membrane is on the outer surface. In the delivery experiment, insulin must permeate via the inner channels in the membrane into the receptor cell that initially contains no insulin. This process implies extensive and prolonged contact of insulin with the inner surface of the membrane. It seems that the surfactant can cover the outer surface of the membrane within a time as short as 30 min. Nonetheless, it cannot occupy the surface of inner channels efficiently in such a short time. Although the membrane containing 35% (w/w) of nanoparticles is much more hydrophilic than the pure ethylcellulose membrane, there still exist some hydrophobic domains in the membrane that are not covered by the nanoparticles. In addition, the hydrophobic groups, such as the isopropyl and methyl groups, in poly(*N*-isopropylacrylamide/methacrylic acid) may also act as hydrophobic surfaces.

Another explanation is that in the delivery experiment, the insulin concentration in the receptor cell is much lower at the beginning than that in the incubation experiment, implying a higher proportion of the monomeric form of insulin. Moreover, due to the limited pore size in the membrane, large molecules with molecular weights of 12 000 or higher could have difficulty in diffusing through the membrane (Yam et al 2000; Wu & Yam 2003; Zhang 2003). These observations suggest that the chance for the dimer and hexamer of insulin to diffuse through the membrane is sparse and thus the monomeric form is the major species that can diffuse across the membrane. As insulin monomer is essential to insulin aggregation, blocking the inner hydrophobic surface is required to prevent insulin from denaturation during delivery.

Conclusions

The stability of insulin delivered through the glucose-responsive polymeric composite membrane is influenced significantly by insulin concentration and by stirring to a lesser extent. Insulin stability can be improved significantly by addition of a non-ionic surfactant in the medium. Pre-saturation of the membrane with the surfactant is necessary for maintaining the stability of insulin that diffuses through the membrane. Therefore, it is believed that insulin can be delivered, with unchanged form, through the glucose-responsive membrane by incorporating a suitable surfactant in the formulation. This study has paved a road for future in-vivo investigation of bioactivity of released insulin and development of an intelligent insulin delivery device that is responsive to changes in glucose concentration.

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