

# A Physicochemical Method for Glucose Controlled Release of Depot Insulin in Vitro

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# Abstract

Proteins, including insulin, can be release from gels made from poly-hydroxyl polymers in response to glucose in the surrounding media in vitro. The release increases with increasing glucose concentration over the range 0 to 200 mM. The result was demonstrated in normal saline at room temperature and artificial plasma at 37°C. The protein containing gels are amenable to injection through narrow gauge hypodermic needles.

# **Keywords**

Diabetes, Insulin, Smart, Depot, Release, Glucose, Responsive, Stimulated

# **1. Introduction**

People with insulin dependent diabetes mellitus (type 1 diabetes) can regulate blood glucose levels by administering insulin. Nevertheless treatment does not lead to normal glucose control, requires multiple injections and testing can result in hypo and hyper-glycemic episodes and can cause long-term complications such as nephropathy, retinopathy and vascular disease. [1]

One way to achieve better blood glucose control is by mechanisms that regulate insulin release or activation in direct response to actual blood glucose concentrations. Insulin pumps can be used. [2] Chemically formulated insulins that respond to glucose have proven elusive.

Described in this paper is a physicochemical method with the potential to allow for insulin release from depots of injected insulin-containing gels or gel particles in response to glucose concentrations in the surrounding body fluids (lymph).

There are many possible permutations of the chemicals used but the basic physicochemical concept involves encapsulating proteins, including the insulin protein or its zinc chelate complex, in fragments or particles of gels or resins made from poly-hydroxyl polymers cross-linked between hydroxyl groups. The cross-linker used here was boron. [3] The integrity of the gel is apparently compromised by the hydroxyl groups on the smaller glucose molecules out-competing those on the much longer polymer components for the cross-linker and as a result the proteins are released into the surrounding media.

# **2. Materials and Methods**

### 2.1. Gel Preparation

Starch gels for exps 3 and 4 were prepared by boiling starch (commercially available tapioca starch) in normal saline (0.9% sodium chloride; saline) containing skim milk which contains about 35 mg/ml sodium caseinate (casein) responsible for the white colour (turbidity). Borax (10%; commercially available, Ward McKenzie Pty Ltd) was mixed into the hot gel and the final gel contained 5.0% starch, 0.75% borax and 3.4% skim milk (a 1:30 dilution equivalent to 0.12% or 1.2 mg/ml casein) in saline. Hot gel (1.0 ml) was dispensed into plastic vials and allowed to set for 1 to 2 hours. The available surface area to volume ratio was 7.0 cm<sup>-1</sup>.

Other polymers such as PVA, plant gum, corn or potato starch were used for some experiments. The polymer used in exp. 5 was a clear plant gum (commercially available for bonding paper). The final polymer concentration in exp. 5 was estimated to be less than 1.0%.

For exp. 1, 30 ml of starch gel was prepared (8.0%) and mixed with 10 ml insulin (Humalog<sup>®</sup>, Lilly, 4.0 mg/ml) [4].

It was cross-linking by mixing in 1.5 ml 10% borax. The final gel contained 6.0% starch, 1.0 mg/ml insulin and 0.75% borax in saline. Aliquots of about 2.0 ml were placed in the bottom of small vials and allowed to set. The available surface area to volume ratio was  $3.5 \text{ cm}^{-1}$ .

#### **2.2. Protein and Starch Elution Experiments**

#### **2.2.1. Experimental Protocols**

For exp. 3, glucose solution (5.0 ml) was added to each vial containing gel and lids were attached. The vials were maintained on the bench at room temperature (nominally  $16^{\circ}$ C). They were mixed by inversion once every 5 min over 1 hr when 1.5 ml supernatant was taken for assays.

The above protocol was repeated for exp. 4 but instead of saline at room temperature (16°C) the experiments were conducted at 37°C using an artificial plasma consisting of 4.0% colloidal gelatin in normal saline. [5] The procedure was as described above but samples and solutions were maintained at 37°C in a water bath. Readings were made while the solutions were warm to ensure the artificial plasma did not solidify. Turbidity was read from a standard curve for skim milk in artificial plasma.

For exp. 1, the vials were randomly divided into three groups and 4.0 ml saline containing 0, 50 mM or 200 mM glucose was added. After 18 hr undisturbed at room temperature the vials were gently rocked and the supernatants poured off and collected for assay.

#### 2.2.2. Insulin Assay

Insulin levels were determined by the BCA protein assay (Pierce) [6]. Samples tested were all adjusted to contain 50 mM glucose because glucose may affect this assay. Absorbance was measured at 562 (610) nm.

#### 2.2.3. Casein Turbidity Assay

Turbidity standard curves were prepared from serial dilutions of skim milk over the range from 0 to 3.0% skim milk (0 to 1.1 mg/ml casein).

The level of casein released into supernatants was determined from the turbidity measured as light transmission through the solution in a basic photometer. White light from a LED was passed through the sample in a glass vial and the voltage produced by a second LED on the other side of the vial measured. Controls were glucose blanks. The casein in the samples was read from the turbidity standard curves. The units used to express turbidity were mg/ml casein.

Skim milk contains 5.2% lactose which means that the gels used had about 5.0 mM of that disaccharide. That was ignored for these experiments.

# 2.2.4. Starch Assay

Supernatant (0.10 ml) was diluted with 1.0 ml water and 1.0 ml iodine solution added. The Iodine Solution contained 2.0% Bentadine® (1% available iodine) solution [7] in 2.0% acetic acid. The solution was used freshly prepared. Glucose and saline blanks controls were not different at these dilutions. The blue colour was measured after 10 min by optical transmission of white light. The starch concentration

in the supernatants was read from a standard curve which was virtually linear for dilution (log scale) against light transmission over the range 0.0001 to 0.1% starch. For exp. 1 absorbance was determined at 610 nm.

#### 2.3. Gel Viscosity and Strength Determinations

For a viscosity standard curve starch gel (12.0%) was prepared in saline containing food dye with heating until boiling. The hot starch was aliquoted into 30 ml vials and mixed with hot normal saline containing the same concentration of food dye to produce samples of 6.0 ml with starch concentrations from 2.0 to 12.0%. Samples were allowed to cool for 2 hr at room temperature and viscosity determined by laying the vials on their sides on a flat surface and measuring the times taken for the gels to run to the edge of the vial. Viscosity was calculated as log (flow rate) and used here as a measure of gel strength.

To determine the effect of glucose on gel strength 4.0 ml of hot starch gel (5.0% starch, 0.5% brilliant blue food dye, 1.0% borax) was mixed into 1.0 ml samples of hot glucose solutions serially diluted in saline in vials to give a final glucose concentration range of 0 to 200 mM. Final starch concentration was 4.0%. Gels were allowed to cool at room temperature for 2 hr before their viscosity was determined. The amount of cross-linking borax (0.8%) used was determined to increase the inherent strength of the gels by the equivalent of about 3 to 5%.

#### 2.4. Other Methods

Glucose was determined using an Accu-Chek® *mobile* blood glucose monitor. [8]

Statistical analysis used standard methods and data is presented as mean+one standard error of the mean. Microsoft Office Excel 7.0 [9] was used for data analysis and graphics.

## 3. Results

#### 3.1. Insulin Released from Cross-Linked Starch Gels by Glucose

In exp. 1 glucose in the surrounding media induced release of insulin from borax cross-linked starch gels, into the supernatants. The release of insulin was significantly higher in the presence of 200 mM glucose ( $0.148\pm0.0055$  mg/ml; n=5; p< $0.001^{***}$ ) than in the zero glucose controls ( $0.113\pm0.002$  mg/ml; n=5). The insulin released in to the 50 mM glucose supernatants fell between the two ( $0.134\pm0.0126$ ; n=5; p<0.10, p>0.10 n.s.). The average amount released was 40% of the calculated equilibrium level, with 30% more released by 200 mM glucose and 19% more by 50 mM glucose, than in the zero glucose controls.

That result was consistent with interference with the gel integrity by glucose as demonstrated by the amount of starch released (solubilized) from the gels into their supernatants by 200 mM glucose ( $4.31\pm0.75$  mg/ml; n=5) compared to the zero glucose controls ( $2.91\pm0.138$  mg/ml; n=5; p<0.005\*\*)

with the 50 mM glucose supernatants values again falling between them  $(3.19\pm0.330 \text{ mg/ml}; n=5; p>0.1 \text{ n.s.}, p<0.05*)$ . That is 48% more and 18% more than the zero glucose controls, by the 200 mM and 50 mM glucose treatments. The starch released averaged 21% of the calculated equilibrium level if all the starch was solubilized.

## 3.2. Gel Viscosity (Strength) Reduced by Glucose

The loss of gel integrity included a softening of the gel (exp. 2). Figure 1 shows that when glucose was mixed into the gel (4.0% starch; 0.8% borax; 0.4% brilliant blue dye plus glucose) during preparation it reduced the gel strength (viscosity). The viscosity fell with increased glucose concentration (0 to 200 mM). Viscosity units used are log (flow rate).



Figure 1. Decrease in gel strength measured as viscosity with increasing glucose concentration during gel formation.

#### 3.3. Release of Casein and Starch from Gels in Saline at Room Temperature (Model System)

## 3.3.1. Casein Release from Starch Gels by Glucose

The model system for most experiments used sodium caseinate (casein) from skim milk (3.4% equivalent to 1.2 mg/ml casein) as the protein in starch gels (5.0%) cross-linked with borax (0.75%) in saline. Whilst casein forms large colloidal complexes it was used for proof of concept and ease of assay and handling. Turbidity is expressed as mg/ml casein.

Figure 2 shows that in exp. 3 casein was eluted from the gel by glucose and under these conditions the elution was glucose concentration dependent (0 to 200 mM). About 48% of the expected casein equilibrium level was eluted into the supernatant in 1 hr by 200 mM glucose in saline at room temperature.



*Figure 2.* Casein elution from gels in saline at room temperature increases with increasing glucose concentration.

#### 3.3.2. Gel Breakdown and Starch Elution in Response to Glucose

Figure 3 shows that similarly for starch solubilization (a measure of gel breakdown) increasing glucose concentration increased starch release from the gel into the supernatants. About 24% of the total starch in the gel was made available for assay in the supernatant over 1 hr at room temperature in saline by 200 mM glucose.

The starch assay was not significantly affected by glucose or borax at these concentrations.



*Figure 3.* Gel breakdown seen as starch elution in saline at room temperature increases with increasing glucose concentration.

#### 3.4. Release of Casein and Starch from Gels Under Physiological Conditions

#### 3.4.1. Casein Elution from Starch Gels by Glucose

Blanks containing glucose over the test range in artificial plasma showed no significant turbidity above the zero glucose blank. Figure 4 shows that, as with saline, casein was eluted from the gel by glucose and the elution was glucose concentration dependent. Further, after 1 hr at 37°C, the conditions used, the casein elution had plateaued for glucose concentrations greater than 50 mM. Below 50 mM glucose the amount of casein eluted appears to follow the glucose concentration.



Glucose concentration (mM)

*Figure 4.* Casein elution from gels in artificial plasma at 37°C increases with increasing glucose concentration.

#### 3.4.2. Gel Breakdown and Starch Release in Response to Glucose

Whilst the natural colour of the artificial plasma did affect the glucose blank readings and there was a slight increase with high glucose the levels did not affect the result. The starch equivalents of glucose in artificial plasma (read from a standard curve using artificial plasma) had an average value less than an acceptable 8.0% of the average test values.



Figure 5. Gel breakdown seen as starch elution in artificial plasma at 37°C increases with increasing glucose concentration.

Figure 5 shows that solubilization of starch from the gel (a

measure of gel breakdown and probably amylose) more or less follows the elution of casein. The starch in the gel was solubilized and made available in the supernatant for assay after 1 hr at 37°C in artificial plasma had plateaued with glucose concentrations greater than about 80 mM. That suggests that much of the integrity of the gel remained but pores were opened up as the cross-linker was sequested by the glucose, allowing elution of the casein. Below that starch solubilisation appears dependent on glucose concentration.

#### **3.5. Additional Comments on the Results**

#### 3.5.1. Osmotic Pressure Not Responsible for the Glucose Effect

The possibility that the glucose effect was due to osmotic pressure is unlikely. Exp. 2 had no supernatant around the gel so the loss of gel strength is a chemical interaction between glucose and the starch or borax. Whilst the gels in exp. 1 did swell slightly over the 18 hours there was no significant difference between the tests or controls  $(0.340\pm0.072 \text{ ml controls}, 0.294\pm0.022 \text{ ml 50 mM and } 0.357\pm0.149 \text{ ml 200 mM glucose})$ . In exp. 4 the gel polymer concentration was virtually the same as the artificial plasma polymer concentration.

#### 3.5.2. Explanations of Other Apparent Anomalies

It should be noted that the different time-frames for exp. 1 (18 hr) and exp. 4 (1 hr) are the consequence of different gel concentrations (6.0% compared to 5.0%) and different mixing regimes (vials static compared to inverted every 5 min).

Also the fact that the maximum elutions of protein and starch in exp. 3 occurred at lower glucose concentrations than in exp. 2 is a consequence of the higher temperature  $(37^{\circ}C \text{ compared to } 16^{\circ}C)$  which is known to reduce the strength of the starch gels used.

#### 3.6. Gel Injectability

In exp. 5 clear gum was used without the need for heating (at room temperature). It was diluted 1:4 and mixed with skim milk and borax to create a viscous soft gel containing 2.5% skim milk (0.9 mg/ml casein) and 0.1% borax in saline. Glucose solutions (2.0 ml) in saline in glass tubes were prepared to cover the range 0 to 200 mM. The soft gel was drawn into a syringe and 0.25 ml injected through a 30 gauge needle into the middle of the glucose solutions. The gel expelled as a long thin stream (surface area to volume 12.8 cm<sup>-1</sup>) that quickly settled as a blob (surface area to volume 7.6  $\text{cm}^{-1}$ ) on the bottom of the tubes. The tubes were incubated undisturbed at room temperature. Samples of the supernatants were taken at 3.5, 5.5 and 24 hr and assayed for casein and then returned to the tubes. The results (Figure 6) show that the casein was slowly released from the injected gels and that the amount release by each time point increased with increasing glucose concentration over the range 0 to 25 mM glucose.



Figure 6. Casein eluted over time from starch gel injected into saline showing the increased release with increasing glucose concentration.

# 4. Discussion

The results presented show that in in vitro experiments proteins such as casein, including insulin, embedded or encapsulated in gels or resins constructed from poly-hydroxyl polymers cross-linked with boron are released by glucose in the surrounding media at a rate greater than that of the diffusion rate in the zero glucose controls. Further, the release is glucose concentration dependent.

The effect appears to be the result of the partial breaking of bonds between polymer molecules and of the pores being opened in the gels by hydroxyl groups in the glucose competing for cross-linking boron molecules.

The non-Newtonian fluid nature of these gels means they are fluid under pressure and hence injectable, but re-solidify after injection.

Further, the effect is reproduced in artificial plasma at 37°C.

One series of experiments using food dye (not reported) suggested that the effect is reversible when the media containing glucose surrounding the gel was replaced by glucose free media. Presumably the glucose molecules in the gel diffuse out more quickly than the boron and some of the original polymer cross-linking re-establishes.

The permutations available for this system are many. Polymers could include, for examples, modified celluloses, pure amylose, plant gums, glycogen or artificial polymers such as PVA or PEG. Agar or maltodextrin do not seem to work easily. Cross-linking molecules other than borax might work; such as poly-acid ester bonds or ketone acetal bonds. Shorter polymers might produce small particles more readily injectable. The existence of any amylases in the lymph is a matter to consider when choosing polymers.

Interestingly and relevantly the polymers suggested are commonly encountered by the immune system and are mostly homopolymers. They are not likely to be particularly antigenic. Further, the gels themselves would offer protection for the embedded proteins (insulin) from macrophages and particularly from lymph proteases (an obvious problem for insulin which is naturally and rapidly degraded by liver proteases in the circulation) which would have to defuse into the gels.

The boron does diffuse out over time but its diffusion rate is retarded because it constantly runs into more hydroxyl groups on the way (effectively an affinity chromatography effect). Therefore there is a non-glucose dependent background release of protein (insulin) similar to that which occurs naturally with islet cells for insulin.

The hydroxyl groups of the aqueous media will eventually take over and disperse and solubilize the whole gel.

## 5. Conclusions

These in vitro studies suggest a potential depot insulin system suitable for glucose controlled release of insulin into the subcutaneous interstitial fluid. Optimizing gel types and characteristics to work efficiently in the glucose physiological range (0 to 30 mM glucose) followed by animal and then clinical trials would be the next steps.

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