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Insulin Delivery Governed by Covalently Modified Lectin–Glycogen Gels Sensitive to Glucose

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Abstract

A glucose-sensitive gel formulation containing concanavalin A and glycogen has been reported previously. Precipitation resulting from the addition of concanavalin A to glycogen has been documented, but the formation of glucose-sensitive gels based on lectin–glycogen interactions is novel and used here in our studies. An improved in-vitro self-regulating drug-delivery system, using covalently modified glucose-sensitive gels based on concanavalin A and a polysaccharide displacement mechanism, is described. The successful use of the covalently modified gels addresses a problem identified previously where significant leaching of the mitogenic lectin from the gel membranes of non-coupled gels was encountered.

Concanavalin A was covalently coupled to glycogen by use of derivatives of Schiff's bases. The resulting gels, like the non-coupled gels, were shown to undergo a gel–sol transformation in response to glucose. Insulin delivery was demonstrated using this covalently modified system in conditions of repeated glucose triggering at 20°C and 37°C. The magnitude of the response was less variable than for the dextran-based gels studied previously.

The performance of this system has been improved in terms of concanavalin A leaching. This could, therefore, be used as the basis of the design of a self-regulating drug-delivery device for therapeutic agents used to treat diabetes mellitus.

Mixtures of the plant lectin concanavalin A with specific polysaccharides have long been known to interact, producing precipitates, because of the interaction of suitably configured terminal and non-terminal sugars with highly specific receptors in the lectin (Goldstein & So 1965; Goldstein et al 1973; Goldstein 1976). It has been shown that under appropriate conditions of hydration and concentration the same process can lead to the formation of glucose-responsive gels, the viscosity of which can be reduced reversibly on interaction with glucose (Taylor 1992, 1993). The gel-switch mechanism depends on competitive displacement of a glucose-bearing polysaccharide by free glucose from concanavalin A. The gel structure which forms between concanavalin A and the polysaccharide is dismantled when glucose causes this displacement. The gel viscosity falls as a result but is restored on removal of glucose, thus providing the switch controlling the diffusion of drug held in a reservoir. Polysucrose (Tanna & Taylor 1994; Taylor et al 1995; Tanna 1996) and dextran (Taylor & Tanna 1994; Tanna 1996) have been used as the polysaccharide components. Others have subsequently used a similar approach to show that polymer-bound glucose and concanavalin A could form a gel undergoing reversible gel–sol transition for the regulation of insulin release (Lee & Park 1994; Obaidat & Park 1996, 1997).

Insulin delivery with these systems has been demonstrated in-vitro (Taylor et al 1995; Tanna 1996). Such glucose-sensitive gels can form the basis of the design of a novel implantable self-regulating drug-delivery device for anti-hyperglycaemic agents and have potential therapeutic value in the management of diabetes. There is a need for such a ''closed-loop'' insulin-delivery device to mimic the physiological pattern of insulin secretion and thereby to minimize the complications of diabetes mellitus (Polonsky et al 1997; Jaremko & Rorstad 1998).

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This study differs from previous studies in that the formulation and insulin-delivery characteristics of gels made with glycogen as the polysaccharide component were examined. Glycogen is also a glucose polymer but has branching characteristics different from those of dextran. The fine structure of glycogen is based on α-1,4 glucosidic links with α-1,6 branching at one in ten (approx.) glucose units. Dextrans have α-1,6 glucosidic bonds with occasional branches formed by α-1,2, α-1,3 and α-1,4 glucosidic linkages, depending on the species of origin. Glycogen has a multiply branched, tiered and ''tree-type'' structure (Manners 1957; Bathgate & Manners 1966; Calder 1991) in comparison with the mainly linear structure of dextran with its short, sparse branches. Because the amount of branching governs the extent of polysaccharide–lectin interactions, it was hypothesized that the resulting gel structure would differ from that of dextran and polysucrose gels, possibly affecting solute delivery when such glycogen-based gels are used in a delivery device. To address a previously encountered problem of significant leaching of the mitogenic lectin from the gel membranes (Tanna & Taylor 1998b), concanavalin A was occasionally coupled covalently to glycogen by use of Schiff's base derivatives. This was achieved by periodate oxidation of glycogen to produce reactive groups that could couple with concanavalin A. The object was to retain the reversible receptor-binding capacity of concanavalin A to glycogen via the terminal glucose units while concanavalin A was permanently anchored to the glycogen at other points. For the purposes of this study the covalent attachment of concanavalin A to aldehydic glycogen would be advantageous because the large covalently coupled macromolecular assembly would not be able to leach through the retaining cellulose nitrate barrier membrane pores, assuming 100% integrity of the structure. A large pore structure is a significant advantage in insulin delivery and it is, therefore, preferable to keep this feature so that the cellulose does not become blocked and rate-determining.

Materials and Methods

Materials

Glycogen (type IX from bovine liver), concanavalin A (type V from *Canavalia ensiformis*), bovine pancreas insulin and sodium metaperiodate ($NaIO_4$) were purchased from Sigma–Aldrich (Poole, Dorset, UK) and sodium borohydride ($NaBH_4$) from FSA Laboratory Supplies (Loughborough, UK). Other chemicals were of reagent grade.

Methods

The method employed for the preparation of the covalently modified glycogen-based gels was that used for the preparation of covalently modified dextran-based formulations (Tanna & Taylor 1997, 1998a). Concentrated stock glycogen solutions (40–60% w/v) were prepared in distilled water or phosphate-buffered saline (PBS) at pH 7·4. Equal volumes of $NaIO_4$ (0·20 M) and glycogen solutions were mixed then stirred in the dark for 24 h to convert the glycogen to the aldehydic form. In a study to examine the effect of the concentration of oxidizing agent, $NaIO_4$ in the range 0·02 to 0·20 M was used. The highly cohesive product resisted dispersion in water during rinsing on a glass sinter. To 1 mL samples of these activated glycogen solutions were added equal volumes of a concanavalin A solution of appropriate concentration and pH. The concanavalin A solutions used were either at uncontrolled pH or at pH 7·4 buffered with PBS. The resulting gel was stirred intermittently at room temperature for 24 h. The resulting Schiff's bases were stabilized by thorough mixing with $NaBH_4$ (1 mg mL^{-1}; 2 mL) for 1 h at 0–4°C. The gels were then rinsed thoroughly with PBS at pH 7·4 on a glass sinter before use. Gels formulated in this way were tested in-vitro for retention of concanavalin A and for glucose responsiveness in terms of differential delivery of insulin.

Insulin solutions 4 mg mL^{-1} were prepared by dissolution in a minimum volume of HCl (0·01 M) and dilution to volume with PBS at pH 7·4 (Brange 1987). For the diffusion experiments a small experimental cell was used to hold a thin layer of a glucose-sensitive gel. In this arrangement the gel was confined between two filter disks (pore size 0·1 μm) to form a barrier membrane for a solute reservoir, while the other side of the gel was exposed to an aqueous temperature-controlled bulk medium to which glucose was added. The gel thickness (pathlength through the gel) was dictated by a spacer gasket between the filters. During each diffusion experiment anhydrous glucose was added to the receptor solution of a test run to produce 2% w/v (111 mM) in the receptor and the output from the reservoir was monitored and compared with a glucose-free control for increase in solute flux in response. The concentration of glucose produced is considerably greater than the upper level of abnormal blood glucose but was used here to ensure each gel was rapidly challenged throughout the 1-mm gel path length used. To create conditions

under which glucose is described as having been removed, the experiment was suspended during replacement with a glucose-free solution matched for temperature, before resuming readings. Solute delivery was monitored spectrophotometrically for insulin at 276 nm. The insulin-release gradients for both test and control were determined, enabling calculation of the ratio of fluxes from the test and control. This was termed the flux factor increase (FI) value. The FI value is a measure of the effect of glucose on solute delivery from the gel system. Earlier glucose dose–response studies with covalently modified glucose-sensitive gels had shown that the glucose sensitivity could be altered by manipulating the pathlength of the gel membrane. Glucose-triggering concentrations between 0·1% w/v (5·5 mM) and 2% w/v (111 mM) gave increasing, graded responses depending on the glucose dose to which the system was subjected (Tanna & Taylor 1998a).

Concomitant release of concanavalin A from the gel membrane was assessed by use of an identical arrangement by using a solute-free reservoir solution. Concanavalin A was assayed at 276 nm and therefore in addition to the use of these data for assessing lectin escape from the gels, they were used to correct the insulin-release profile.

Results and Discussion

Depending on concentration and hydration conditions, precipitates and glucose-sensitive gels resulted when periodate-oxidized glycogen was mixed with concanavalin A. Gels could be formulated with much lower concentrations of concanavalin A in comparison with the covalently modified dextran-based gels studied (Tanna & Taylor 1997, 1998a). Differences in the availability of terminal glucose units for dextran and glycogen might be responsible for this behaviour. The covalently modified gels, like the uncoupled counterparts were shown to undergo gel–sol transformations as a function of glucose content. This is evidence that after covalent coupling the lectin receptor sites have retained their capacity to interact reversibly with glucose, implying that free glucose could still displace the glycogen terminal units, dismantling the temporary cross-linking between the lectin. Two main types of interaction are therefore proposed to occur in a covalently modified gel—the lectin–polysaccharide receptor interaction (physical cross-linking) and interactions associated with the chemical cross-linking of glycogen and concanavalin A, because of the formation of a Schiff's base between the NH_2 groups of concanavalin A and glycogen dialdehydic derivatives. Reduction of the Schiff's base led to a combination in which the concanavalin A is fixed via a permanent bond. The gel formulations could only transform between gel and sol in response to glucose if the lectin receptor sites remain freely accessible, if the terminal glucose units of glycogen remain undamaged and if the gel network is not so tight that it impedes glucose diffusion. Dismantling of the physical cross-linking caused by the competitive displacement of glycogen by glucose from the lectin receptor sites would result in a gel–sol transformation. As a consequence a change in the complexity of the gel structure would arise from a densely cross-linked narrow-channelled gel to a weakly cross-linked wider-channelled sol.

Concanavalin A retention

"Insulin-free" control experiments revealed that for all the formulations studied concanavalin A escape was restricted compared with non-coupled gels (Figure 1). Several factors influenced the efficacy of covalent coupling of concanavalin A to glycogen, including the concentration of oxidizing agent, the buffering of glycogen in PBS before oxidation, the pH of the concanavalin A solution before coupling, the concentration of oxidized glycogen and the concentration of concanavalin A.

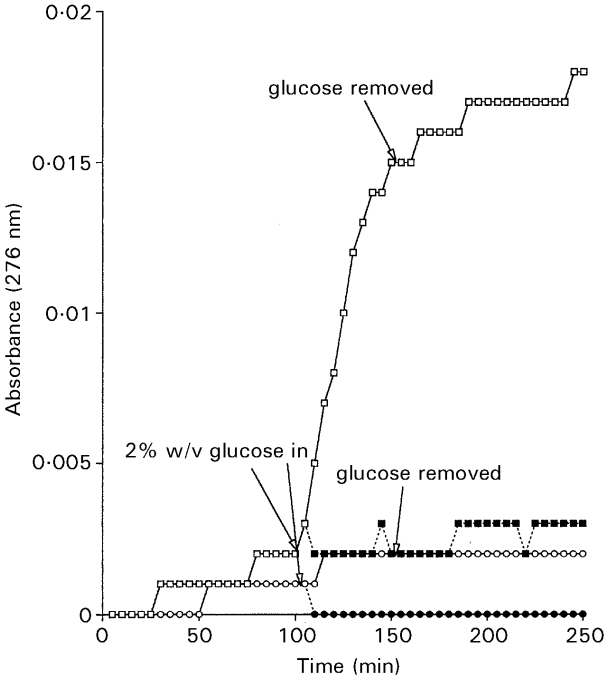

Figure 1. Comparison of the concanavalin A-release profiles of an uncoupled 20% glycogen–5% concanavalin A gel and a coupled 20% glycogen (oxidized)–5% concanavalin A gel. □ Test uncoupled gel (glucose-triggered), ■ control uncoupled gel (glucose-free), ○ test coupled gel (glucose-triggered), ● control uncoupled gel (glucose-free).

For the covalently modified gels, as the concentration of the oxidized glycogen stock preparation was increased more concanavalin A was retained within the glucose-induced sol state. It was envisaged that because of the compact inner structure of glycogen, steric factors would prevent concanavalin A from accessing all possible terminal glucose units. This was based on information that the use of specific enzymes for structural analysis of glycogen has shown that the interior of glycogen is less susceptible to enzymatic attack because of steric hindrance (Manners 1957; Marshall 1974; Calder 1991). Therefore, it is possible that increasing the glycogen concentration increased the availability of susceptible active sites on the outer tiers to which the concanavalin A could couple, thus enabling better chemical cross-linking. Reducing the concentration of oxidizing agent from 0·2 to 0·02 M resulted in greater escape of concanavalin A, probably because the number of oxidized glycogen sites was insufficient for covalent bonding of all protein within the structure. Concanavalin A retention was greater in gels coupled using media buffered at pH 7·4 than in those at pH 5·9; the capacity to respond to glucose by sol formation was shown by all formulations. Schacht et al (1993) discussed the effect of pH on the cross-linking of gelatin by dextran dialdehyde. Effective chemical cross-linking involved unprotonated amines and was accomplished when the pH approached or exceeded the isoelectric point of the protein. Values of the isoelectric point reported for concanavalin A range from 4·5 to 8 (Becker et al 1976). It is feasible, therefore, that the pH 7·4 system coincides with the isoelectric point for a particularly relevant lysine (un-ionized) in concanavalin A, thus favouring coupling. It is possible that pH or ionic strength induces changes in glycogen, leaving it vulnerable to NH_2 groups of concanavalin A during the coupling process.

Insulin transport in covalently modified gels

In-vitro experiments demonstrated the delivery of insulin by using a covalently modified glycogen-based gel at 20°C (data not shown) and 37°C (Figure 2). The glucose-provoked FI values were similar for both temperatures although the contributory flux values were higher at 37°C. For the glucose response to be intact the covalently modified glycogen-based gel system must have avoided the interactive parts of the mechanism, probably because of protection by engaged terminal glucose units. Despite the complexity of the multiply branched glycogen-based gels in comparison with the dextran-based gels, the gel structure was loose enough for delivery of insulin. For the glycogen-based gels it is envisaged that the individual structures are so dense that insulin diffuses through the interstices between particles, whereas in loosely and homogeneously cross-linked dextran-based gels insulin is probably more likely to diffuse through the structure. In repeated glucose-trigger experiments comprising five glucose challenges and removals, analogous to the covalently modified dextran-based gel systems examined previously (Tanna & Taylor 1997, 1998a), glucose-induced increased release of insulin with the flux reverting to a low level on removal of glucose from the receptor solution, was observed on each cycle with the glycogen-based system (Figure 3). The resulting FI values were of a similar order of magnitude (Figure 4), and thus more reproducible and lower than for the lectin–dextran conjugate gels studied previously (Tanna & Taylor 1998a).

Conclusions

A polymeric gel formulation containing concanavalin A and glycogen is reported here because of its glucose sensitivity and its in-vitro insulin delivery when, as a thin layer, it forms the rate-determining membrane of a solute reservoir. The performance of such gel formulations in terms of concanavalin A leaching was significantly improved in the modified gel formulations in which

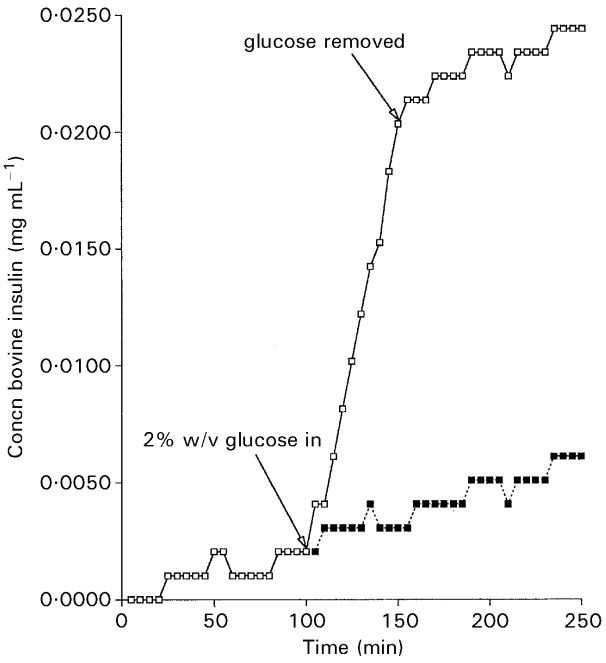

Figure 2. Insulin release profile, at 37°C, across a covalently modified 20% glycogen (oxidized)–5% concanavalin A gel formulation. □ Test (glucose-triggered), ■ control (glucose-free).

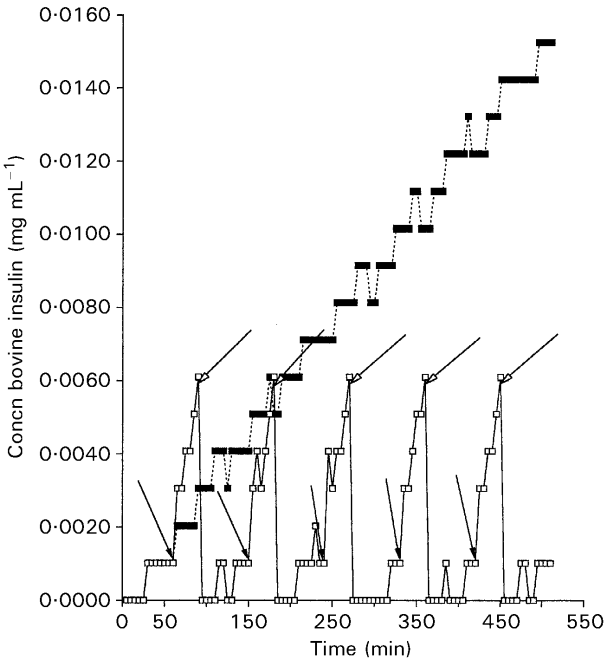

Figure 3. Insulin release profile, at 37°C, across a covalently modified 20% glycogen (oxidized)–5% concanavalin A gel, under conditions of repeated glucose triggering. □ Test (glucose-triggered), ■ control (glucose-free). The solid arrows indicate input of 2% (w/v) glucose; the open arrows indicate removal of glucose.

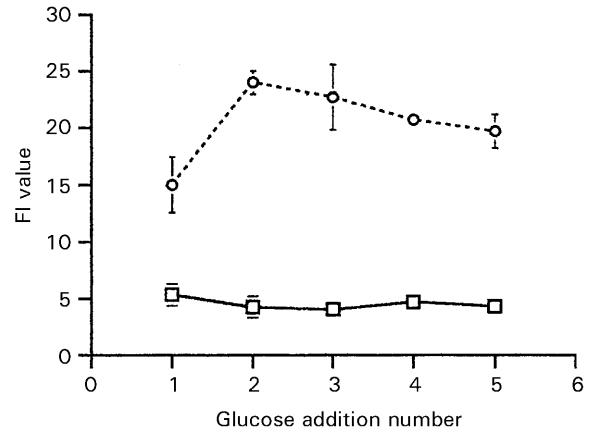

Figure 4. The magnitude of the glucose response upon repeated glucose triggering of a covalently modified glycogen–lectin gel system (○) compared with a dextran–lectin gel system (□).

the lectin is covalently linked to glycogen by the periodate method. Despite the structural and size differences of glycogen and dextran, glucose sensitivity was observed for the glycogen-based gels. Such differences could have design implications for the architecture of a hydrogel. The performance of this gel system was investigated using a bovine insulin reservoir system. Chemically unmodified insulin could be delivered differentially in a specific response to glucose by using a glucose-sensitive gel membrane containing concanavalin A covalently coupled to glycogen. In this design the increase in insulin delivery has been shown to be reversible and could be triggered repeatedly by glucose. Reproducibility in the magnitude of the glucose-provoked response could be advantageous in any future clinical use of such gels as the basis of a self-regulating anti-hyperglycaemic delivery device. The feasibility of the novel gel formulation as the basis for the design of a self-regulating drug delivery device for therapeutic agents used to treat diabetes mellitus has been highlighted. This system has advantages over some other self-regulating dosage prototypes that have necessitated the derivatization of insulin to bear a sugar structure which then participates in competition for receptors on concanavalin A (Sato et al 1984; Pai et al 1992; Baudys et al 1995), because of the applicability to unmodified insulin or other antihyperglycaemics if desired.

Further development work will entail the use of polymeric carriers for the mechanism components as an alternative means of addressing the problem of component leaching. In addition the issue of insulin stability in the device must be addressed to facilitate the development of the self-regulating drug-delivery device for in-vivo use.

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