Insulin-Responsive Glucagon Delivery for Prevention of Hypoglycemia

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***H**ypoglycemia, the state of abnormally low blood glucose level, is an acute complication of insulin and sulfonylurea therapy in diabetes management. Frequent insulin dosing and boluses during daily diabetes care leads to an increased risk of dangerously low glucose levels, which can cause behavioral and cognitive disturbance, seizure, coma, and even death. This study reports an insulin-responsive glucagon delivery method based on a microneedle (MN)-array patch for the prevention of hypoglycemia. The controlled release of glucagon is achieved in response to elevated insulin concentration by taking advantage of the specific interaction between insulin aptamer and target insulin. Integrating a painless MN-array patch, it is demonstrated that this insulin-triggered glucagon delivery device is able to prevent hypoglycemia following a high-dose insulin injection in a chemically induced type 1 diabetic mouse model.*

Insulin replacement is essential for type 1 and advanced type 2 diabetic patients.[1,2] For patients with type 1 diabetes in particular, intensive insulin therapy is associated with improved glycemic control and decreased risk of long-term complications.[3,4] However, frequent insulin dosing and boluses, either through injection or through subcutaneous insulin infusion, leads to an increasing risk of hypoglycemia, or dangerously low levels of glucose in the blood. Episodes of hypoglycemia are characterized by behavioral and cognitive disturbance, and if untreated, can progress to seizure, coma, and even death.[5] Despite of the treatment advances in the electronic/mechanical devices and chemical approaches to insulin delivery, hypoglycemia still remains a concern, even in a closed-loop insulin delivery system.[6] Glucagon, a peptide hormone produced by alpha cells of the pancreas, works to counteract the effect of insulin and raises blood glucose levels (BGLs). The pancreas releases glucagon when BGLs are low, which acts in the liver to promote conversion of stored glycogen into glucose that is released into the bloodstream.[7,8] Thus, glucagon is effective for prevention and treatment of hypoglycemia, and several dual hormone (insulin and glucagon) infusion pumps with continuous glucose monitoring systems have been developed to improve blood glucose control.[9,10] However, the lag in glucose feedback and biofouling still limit the further clinical applications of these electronic/mechanical devices.[2,11]

Here, we report a novel, insulin-responsive glucagon delivery strategy incorporating a microneedle-based transdermal patch for prevention of hypoglycemic episodes during diabetes management. To achieve insulin-responsiveness, an insulin aptamer comprised of a single-stranded oligonucleotide with unique secondary structure that can specifically bind to its target insulin[12,13] was conjugated to glucagon. The aptamer–glucagon conjugates (Apt–Glu) were further

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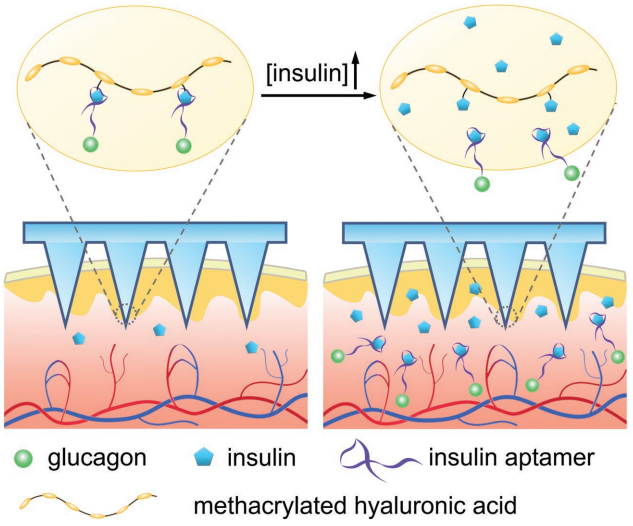

Figure 1. Schematic of the insulin-responsive glucagon delivery system based on MN-array patches.

bound to insulin immobilized on methacrylated hyaluronic acid (*m*-HA) through the interaction between insulin aptamer and insulin. HA was chosen due to its high biocompatibility and biodegradability.[14] The insulin-responsive glucagon conjugated HA (Glu–HA) matrix can be formed via polymerization with crosslinkers and photoinitiator post UV irradiation.[15] In the presence of a high insulin concentration, glucagon can be rapidly released from the HA matrix through the competitive binding between free insulin and immobilized insulin on HA. For a long-term, painless, and convenient treatment,[16–19] the insulin-responsive Glu–HA matrix was further integrated with a microneedle (MN)-array patch for transcutaneous administration. The MNs formed from Glu–HA were able to release glucagon in response to the elevated interstitial fluid insulin level in vascular and lymph capillary networks, thereby preventing the risk of hypoglycemia (**Figure 1**). Finally, we demonstrated this insulin-triggered glucagon delivery system plays a remarkable role in prevention of hypoglycemia after injection of a high dose of insulin in a streptozotocin (STZ)-induced type 1 diabetic mouse model.

In order to prepare insulin-responsive Glu–HA, we first synthesized insulin conjugated *m*-HA (Ins–HA) and insulin aptamer modified glucagon (Apt–Glu). The covalent coupling of insulin onto *m*-HA was achieved by formation of an amide bond between the primary amino groups of insulin and the carboxylic acid groups of the *m*-HA. The Apt–Glu conjugate was obtained from the amino-modified aptamer and thiolated glucagon via a hetero-bifunctional linker. The successful modification on glucagon was clearly identified by the matrix-assisted laser desorption/ionization mass spectrometry (Figure S1, Supporting Information). The bioactivity of the resulting Apt–Glu conjugates was confirmed upon administration on the healthy mice when compared to the native glucagon (Figure S2, Supporting Information). After coincubating Apt–Glu and Ins–HA in Tris buffer (50×10^{-3} M Tris–HCl, 10×10^{-3} M KCl, 100×10^{-3} M NaCl, pH 8.0), Apt–Glu bound to Ins–HA to form insulin-responsive Glu–HA through specific interaction between the binding aptamer and target insulin.

Upon the addition of a crosslinker *N*,*N*′-methylenebisacrylamide and a photoinitiator, Glu–HA can form a hydrogel by photopolymerization after UV irradiation for a short period of time. To assess the ability of Glu–HA to respond to insulin, the prepared hydrogels were incubated with 1× phosphate buffered saline (PBS) [137×10^{-3} M NaCl, 2.7×10^{-3} M KCl, 10×10^{-3} M Na_2HPO_4, 2×10^{-3} M KH_2PO_4 (pH 7.4)] at increasing insulin concentrations. As presented in **Figure 2**a, the release rate of glucagon from the Glu–HA hydrogel was highly dependent on the concentration of insulin, such that the hydrogel incubated with 1 mg mL^{-1} insulin showed the fastest release rate compared to those with 0, 0.1, and 0.5 mg mL^{-1} insulin. The insulin responsiveness of the Glu–HA hydrogel was further verified by rapidly changing the insulin concentration in the solution and measuring glucagon release rate. The Glu–HA hydrogel was stable in PBS buffer without insulin. When adding 1 mg mL^{-1} insulin to the solution, the Glu–HA hydrogel quickly released glucagon (Figure 2b). We attributed this remarkable insulin-responsive release of glucagon to the competitive binding between free insulin and insulin conjugated on *m*-HA to their specific aptamer on the Apt–Glu conjugate.

A MN-based transdermal delivery system was chosen to achieve a painless, convenient, and long-term administration.[16,17] In order to fabricate drug-loaded MN, Glu–HA, the crosslinker *N*,*N*′-methylenebisacrylamide and a photoinitiator were first loaded in the tip region of a silicone mold of MN-array patch through centrifugation. After exposure to UV irradiation for 60 s, the HA matrix containing glucagon was formed by photo-crosslinked, which can not only avoid the diffusion of Glu–HA in vivo, but also enhance the stiffness of MNs for penetration. The prepared MNs were arranged in a 15 × 15 array (**Figure 3**a). Each MN is 300 µm in diameter at the base and 600 µm in height (Figure 3c). To further confirm the distribution of Glu–HA in the MNs, a fluorescein isothiocyanate (FITC)-labeled Glu–HA loaded patch was imaged by fluorescence microscopy. As shown in Figure 3b, Glu–HA was mainly located in the tips of MNs. Using a

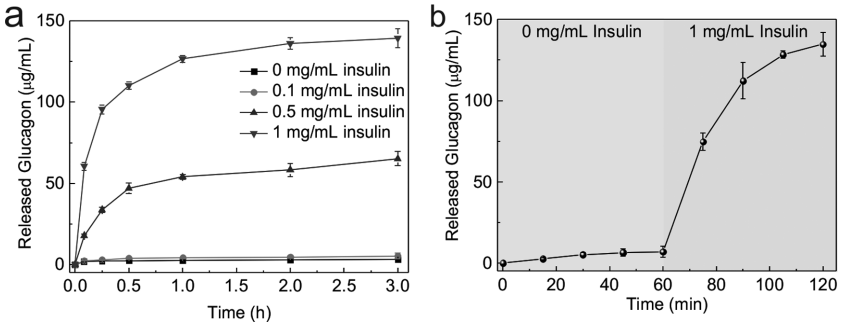

Figure 2. a) In vitro accumulated glucagon release from the Glu–HA hydrogel in increasing concentrations of insulin at 37 °C. b) Self-regulated profiles of the Glu–HA hydrogel present the release rate of glucagon as a function of insulin concentration.

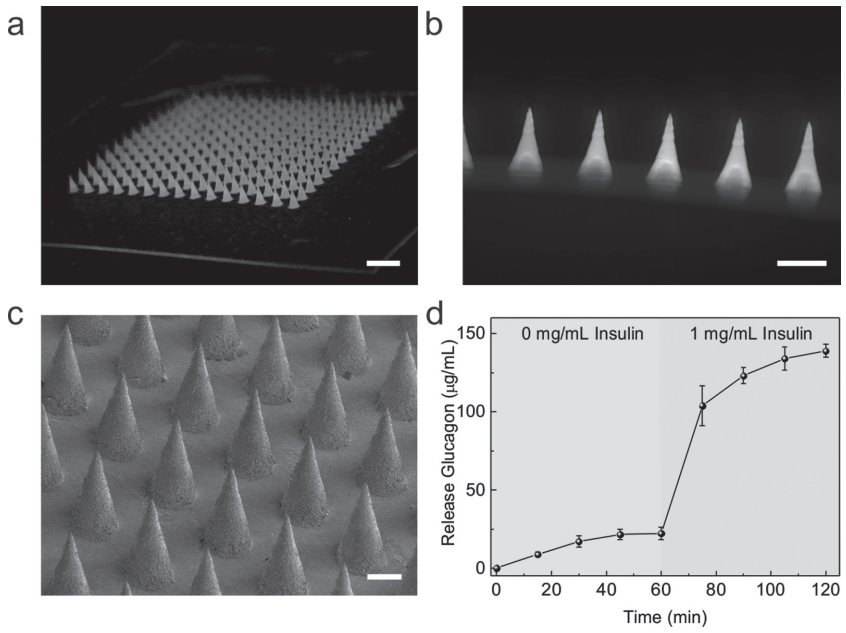

Figure 3. a) A photograph of the MN-array patch. Scale bar is 1 mm. b) A fluorescence microscopy image of rhodamine-labeled MN loading FITC-labeled Glu–HA. Scale bar is 500 μm. c) An SEM image of MN array. Scale bar is 200 μm. d) Self-regulated profiles of the Glu–HA loaded MN present the release rate of glucagon as a function of insulin concentration.

tensile compression machine, the failure force for crosslinked MN was determined to be 2.8 N per needle (Figure S3, Supporting Information), which is sufficient to penetrate skin without breaking.[20] The Glu–HA loaded MNs exhibited similar insulin-responsive capability compared to the hydrogel (Figure 3d).

Next, the in vivo insulin-responsive performance of Glu–HA loaded MNs were tested in an STZ-induced type 1 diabetic mouse model. Prior to a subcutaneous injection of insulin, the drug loaded MN-array patch was administered on the dorsum of mice. The successful penetration of MNs was affirmed by the trypan blue staining of dorsum skin (Figure S4a, Supporting Information). In addition, the hematoxylin and eosin staining result showed the MN could be removed intact from skin (Figure S4a, Supporting Information), indicating its minimal side effect. Mice were injected with a high dose of insulin sufficient to cause profound hypoglycemia and the BGLs of the treated mice were monitored over time. As present in **Figure 4**a, the BGLs of the mice in control group rapidly decreased to normal range (<200 mg dL^{-1}) within 0.5 h, and continued to decline to a hypoglycemic state (<70 mg dL^{-1}).[21] The long-term hypoglycemia caused the death of the mice. By contrast, the BGLs of the mice with Glu–HA loaded MNs were maintained at 80 mg dL^{-1} for 2 h, and then slowly returned to hyperglycemia, which indicated the Glu–HA loaded MNs were able to respond to high insulin level and release glucagon into the regional lymph and capillary vessels to avoid the risk of hypoglycemia. As expected, the serum glucagon levels in mice administered with Glu–HA loaded MNs significantly increased following the increase of insulin concentration (Figure 4c; Figure S5, Supporting Information).

To further assess the responsiveness and the potential for inappropriate release of glucagon by Glu–HA loaded MNs, the mice with and without patches were subcutaneously injected with a low dose of insulin. The BGLs of mice in both groups declined to normal state and began to increase 2 h postinjection (Figure 4b). Finally, the BGLs returned to hyperglycemia within the similar time period. The mice with MNs also presented reduced serum glucagon levels compared to those injected with the high dose of insulin, suggesting that glucagon release was directly responsive to serum insulin levels (Figure 4c). Finally, Glu–HA loaded MNs were tested on healthy mice. Unlike the free glucagon-loaded MN, which led to rapid increase in BGLs due to the burst release of glucagon, the mice treated Glu–HA loaded

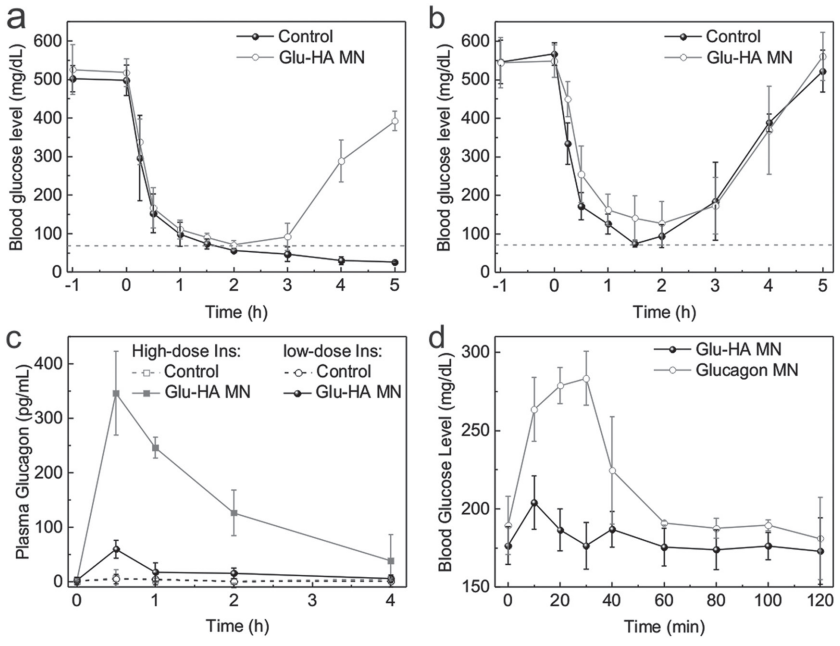

Figure 4. In vivo studies of the Glu–HA loaded MN-array patches for prevention of hypoglycemia. a) BLGs in STZ-induced diabetic mice with or without Glu–HA loaded MN after injecting a high-dose insulin (100 μg). b) BLGs of mice with or without Glu–HA loaded MN after injecting a low-dose insulin (20 μg). Hypoglycemic levels (<70 mg dL^{-1}) were indicated by the dashed line. c) Plasma glucagon concentrations in treated mice. d) The blood glucose changes of healthy mice administered with Glu–HA loaded MN or free glucagon-loaded MN.

MNs did not show significant changes in BGLs (Figure 4d), indicating there was little leak in Glu–HA MNs in healthy mice. Since HA is highly biocompatible and biodegradable, no significant inflammation was observed around the region 24 h postadministration of Glu–HA MN (Figure S6, Supporting Information). Collectively, the Glu–HA loaded MNs have capability to release glucagon in a serum insulin level dependent manner to prevent hypoglycemia in the insulin replacement therapy.

In summary, we developed an MN-array patch-based approach for insulin-triggered delivery of glucagon. Through the competitive binding between free insulin and immobilized insulin on HA to insulin aptamer-modified glucagon, the drug-loaded HA matrix can effectively release glucagon under a high insulin concentration, but does not release glucagon without insulin. In vivo studies in a type 1 diabetic mouse model demonstrated that the insulin-responsive MN-array patch was able to prevent hypoglycemia after injection of a high dose of insulin sufficient to cause hypoglycemia, while showing insignificant action in mice treated with a low dose of insulin. The potential translation of this glucagon patch would profoundly improve the health as well as the quality of life of type 1 and advanced type 2 diabetic patients by both facilitating insulin intensification with reduced risk of hypoglycemia and preventing morbidity and mortality from severe episodes of hypoglycemia. This aptamer-incorporated controlled release method can also be extended to engineer other closed-loop therapeutic delivery systems[22] to treat a variety of other diseases.

Experimental Section

Materials: All chemicals were purchased from Sigma-Aldrich unless otherwise specified and were used as received. Human recombinant insulin (Zn salt, 27.5 IU mg^{-1}) was purchased from Life Technology. Amino modified insulin binding aptamer was purchased from Integrated DNA Technologies Inc. (Coralville, IA, USA): 5′-H_2N-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC-3′.

Synthesis of Apt–Glucagon: Glucagon or FITC-labeled glucagon was thiolated by reacting with the Traut's Reagent (2-iminothiolane, Pierce) in PBS (pH 8.0) at a molar ratio of 1:10 for 1 h at room temperature (RT). Excess Traut's Reagent was removed using a centrifugal filter device (molecular weight cut-off = 3 kDa). In the meantime, amino modified insulin aptamer was mixed with sulfosuccinimidyl-4-(*N*-maleimidomethyl)- cyclohexane-1-carboxylate (Sulfo-SMCC, Pierce) in PBS (pH = 7.4) at a molar ratio of 1:5 for 0.5 h at RT. The activated aptamer was purified using a Nap-5 column. Finally, the purified aptamer and glucagon-SH were mixed in PBS (pH 8.0) at a molar ratio of glucagon:aptamer = 1:2. After 24 h reaction at 4 °C, the obtained Apt–Glu was purified using a Nap-5 column, and stored at 4 °C until use.

Synthesis of Ins–HA: 60 mg of *m*-HA was dissolved in water, to which 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (0.19 g) and *N*-hydroxysuccinimide (0.13 g) were added and stirred for 15 min at RT. Then insulin (30 mg) was added to the mixture and reacted at 4 °C for 24 h. The reaction solution was thoroughly dialyzed against DI water for 1 d. Finally, Ins–HA was obtained by lyophilization. M_n = 431 475 g mol^{-1}.

Preparation of Glu–HA Hydrogel: Crosslinker *N*,*N*′-methylenebisacrylamide (w/v = 2%) and photoinitiator (Irgacure 2959, w/v = 0.5%) were mixed in Glu–HA solution. After UV irradiation (wavelength: 365 nm) for 60 s, the mixture underwent the crosslinking polymerization to form the hydrogel.

In Vitro Release Studies: After preparation of the FITC-labeled Glu–HA hydrogel, various PBS solutions with 0, 0.1, 0.5, and 1 mg mL^{-1} insulin were added to each tube and incubated at 37 °C on an orbital shaker. At predetermined time points, the sample was centrifuged (8000 rpm, 30 s) and 100 µL of the supernatant was removed for analysis by measuring the emission intensity of FITC at 519 nm with the excitation wavelength at 495 nm.

In Vivo Studies Using STZ-Induced Diabetic Mice: The in vivo performance of MN-array patches was evaluated on STZ-induced adult diabetic mice (male C57B6, Jackson Lab, USA). The animal study protocol was approved by the Institutional Animal Care and Use Committee at North Carolina State University and University of North Carolina at Chapel Hill. The plasma-equivalent glucose was measured from tail vein blood samples (≈3 µL) of mice using the Clarity GL2Plus glucose meter (Clarity Diagnostics, Boca Raton, Florida). Mouse glucose levels were monitored for two days before administration, and all mice were fasted overnight before administration. Five mice for each group were selected to be transcutaneously treated with blank MNs containing only *m*-HA or Glu–HA loaded MNs. Afterward, a high-dose insulin (100 µg) or a low-dose insulin (20 µg) was subcutaneously injected into each mouse. The glucose levels of each mouse were monitored over time. In order to measure the plasma glucagon concentration in vivo, 25 µL of blood sample was drawn from the tail vein of mice at indicated time points. The serum was isolated and stored at −20 °C until assay. The plasma glucagon concentration was measured using Human Glucagon ELISA Kit (Thermo Scientific).

Statistical Analysis: All results presented are mean ± s.d. Statistical analysis was performed using Student's *t*-test or ANOVA test. With a P value <0.05, the differences between experimental groups and control groups were considered statistic.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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