Protein Delivery

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Construction of a Photoactivated Insulin Depot**

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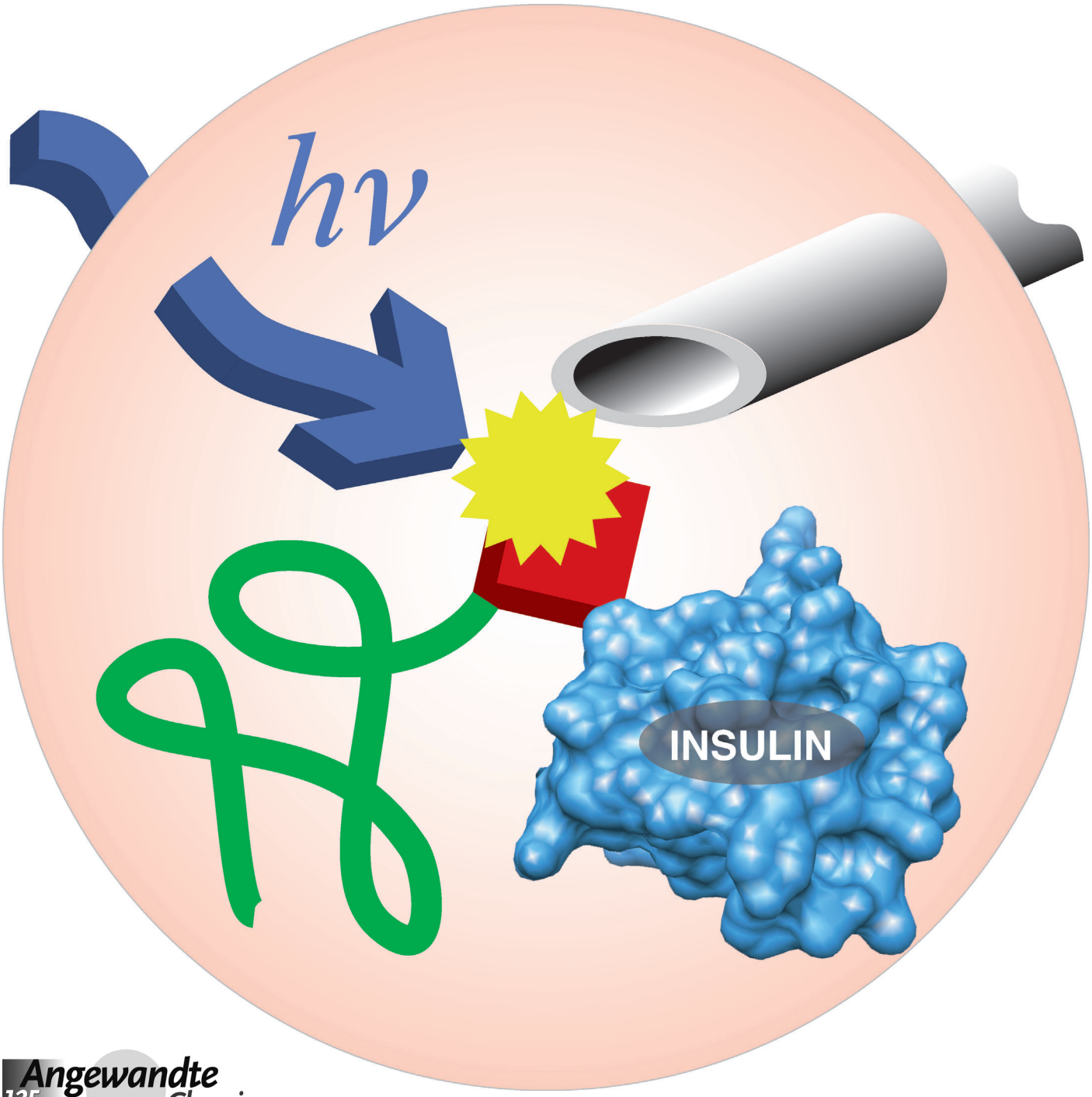

Insulin is a primary tool for the treatment of type I diabetes.[1] However, multiple challenges accompany its administration. Because of low oral bioavailability, it is typically injected multiple times per day, a significant lifetime burden on patients. Moreover, because of fluctuating blood sugar levels, the required amounts of insulin continuously vary over the course of a day. A current solution to the challenge of multiple daily injections is the use of an insulin pump, which delivers insulin transdermally through a cannula, an awkward and limiting solution.[1]

We have conceived of a less invasive approach, in which light is used to activate the release of insulin from a covalently linked depot (Figure 1). The elements of the system are a polymer, a photocleavable linker, and the therapeutic, insulin. Ideally, the polymer should be insoluble, so that it remains at the site of injection, and biodegradable, so that after the majority of the insulin has been released, the polymer can be cleared from the system.

Insulin has features that make it particularly amenable to the photoactivated depot (PAD) approach. Because blood sugar levels vary greatly, the required concentrations of insulin in the blood also vary greatly from hour to hour. Moreover, the total volume of insulin required in a day is small, on the order of 1 mg, the equivalent of 1 μL. Even with a 10:1 ratio of carriers to insulin in a PAD, a typical 250 μL injection of insulin would contain the equivalent of 25 days insulin. The principle challenge is to engineer a material in which insulin maintains its integrity during the synthetic process, and is retained effectively until light releases it in a precisely metered fashion and in its native form.

We have successfully made this material by using a new azide derivative of the 1-(4,5-dimethoxy-2-nitrophenyl)ethyl (DMNPE) group[2] that reacts with insulin. The final link to the insoluble resin is made through a "click"-type reaction with a strained cyclooctyne (DBCO) that is resin-bound. We demonstrate that insulin modified with both one and two DMNPE–azide moieties is photoreleased in a similar fashion, with the dimodified species being photolyzed in two sequential reactions, each of which has identical kinetics. This suggests chemically similar sites of modification. Furthermore, we demonstrate that the insulin modified with DMNPE–azide efficiently reacts with resin-bound DBCO, and that this final material shows efficient and metered photorelease of native insulin when using a 365 nm light-emitting diode (LED).

We are pursuing multiple ways of making the photocleavable link between insulin and an insoluble resin. One of the most direct ways of accomplishing this is using a "click" approach to make the final link between resin and insulin (Figure 2). We started from the known DMNPE derivative **2**, which contains both carboxy and ketone groups.[3] This was modified with an amino azide **1** to make the corresponding amide **3** in good yield. The ketone group was then transformed into the hydrazone **4** by using hydrazine, and finally converted to the diazo derivative **5** by using MnO_2, a process based on previously developed DMNPE chemistry in our group.[4] We found that a parallel approach, in which propargyl amine was condensed with **2**, was not effective.

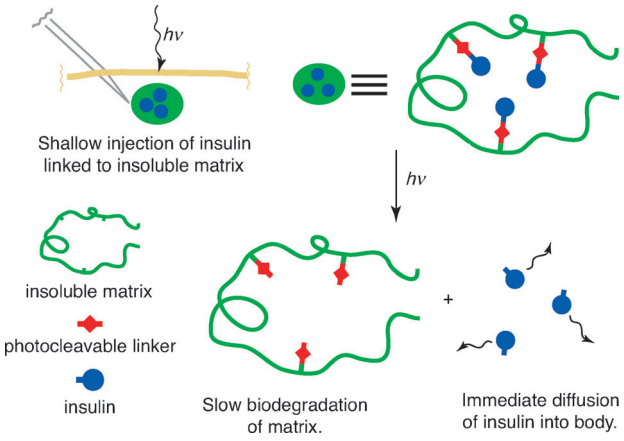

Figure 1. Overall approach to the photoactivated depot (PAD). A drug, insulin in this case, is linked to an insoluble but biodegradable resin, through a photocleavable linker. The conjugate is injected in a shallow depot cutaneously or subcutaneously. Irradiation breaks the link of insulin from the resin, thereby allowing it to diffuse away from the resin and be absorbed by the systemic circulation. Ultimately the resin is biodegraded.

The diazo–DMNPE–azide **5** was reacted with insulin directly in DMSO, using a 2:1 mole ratio. HPLC analysis of the reaction mixture shows two main, well-resolved peaks as well as a minor one (Figure 3a). The compounds in these peaks were isolated and identified by using ESI–MS as unreacted insulin (58%), monomodified insulin ("insulin monoazide" **6**, 32%), and dimodified insulin ("insulin diazide" **7**, 9%). A less highly modified sample was prepared for the resin modification experiments using a 1:1 ratio of insulin to **5**. This reaction product showed 75% unreacted insulin, 23% insulin monoazide **6**, and 2% insulin diazide **7**. For the resin linking, this mixture was used directly, because the insulin was expected to be "silent" during the coupling, thus leaving principally insulin monoazide **6** to react with the resin.

For our studies, we have used a ChemMatrix rink amide poly(ethylene glycol) (PEG) resin. In the long term, we are interested in using a biodegradable matrix, so that the depot can be cleared from the site after a majority of the insulin has been released. The use of the PEG rink amide resin gave us the ability to analyze intermediates through TFA-induced cleavage as well as through final photolysis.

To link insulin monoazide to the resin, we first attempted a copper(I)-based click approach, in which the resin was derivatized with propargyl amine. This proved unsuccessful

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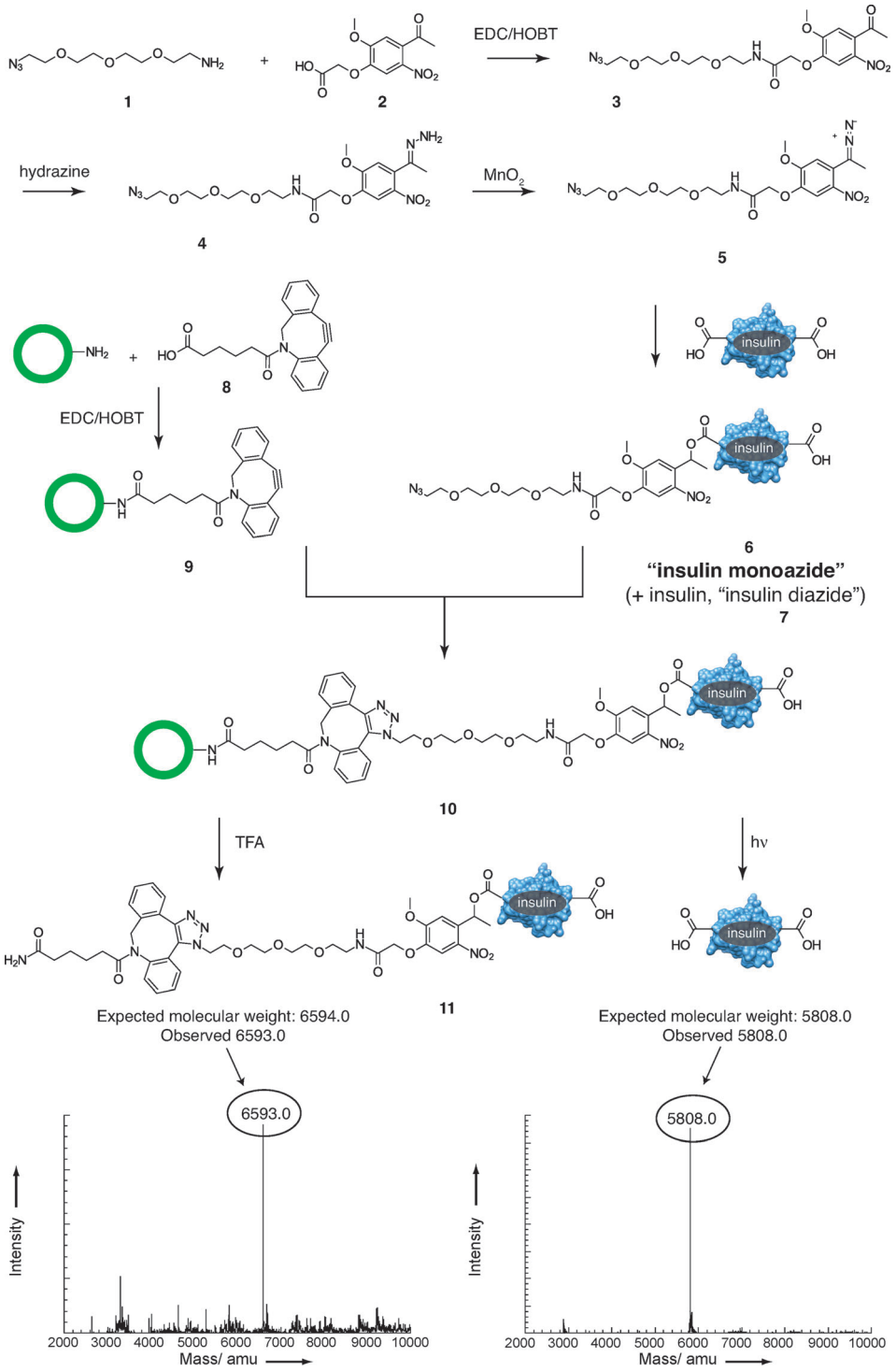

Figure 2. Synthesis and characterization of the photoactivated depot. The depot is constructed in a convergent fashion, with the alkyne portion being attached to the resin, and the azide being attached to the insulin through a photocleavable linker. Copper-free click conditions make the final link between insulin and resin. TFA-induced cleavage of the total conjugate from the resin confirms the covalent nature of the linkage, while photolysis confirms the release of unmodified, native insulin. HOBT = 1-hydroxybenzotriazole, EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

when using a variety of conditions (data not shown).[5] We then turned to the strained cyclooctyne DBCO **8**, which was efficiently reacted with the resin amine directly to form **9** (Figure 2).[6]

Reaction of insulin monoazide with the resin-bound DBCO was monitored by examining the loss of insulin–azide from the supernatant surrounding the resin. While this test sample was being prepared, an analogous control sample was prepared in which native insulin was added to DBCO-modified resin. The purpose of this control was to insure during photolysis experiments that the generation of native insulin was due to actual photolysis and not simply loss of adsorption.

The resulting materials were analyzed in multiple ways. To confirm the adduct and link were as intended, the modified resin was treated with 95% TFA to cleave the carboxamide link. The material that was released from the resin was analyzed by ESI–MS and showed a single prominent mass (6593.0), similar to the mass expected of the insulin–DMNPE–triazole–DBCO adduct **11** (6594.0). A second sample of the modified resin was irradiated using a 30 W black-ray fluorescent fixture. The supernatant as analyzed showed a single peak in the HPLC with retention time consistent with a native insulin standard. This was confirmed by ESI–MS analysis (mass expected 5808.0, mass observed 5808.0; Figure 2).

We examined the kinetics of photolysis in two ways: in the isolated and purified insulin mono and diazides (**6**,**7**), and in the final modified resin. The former experiment provides us with limiting values of photolysis, as the system is homogenous. The latter is closer to "real-world" conditions, but is complicated by the inherent variability of light propagation through inhomogenous resin. Furthermore, solution photolysis of the mono and diazides gives us insights into the site of attachment of the DMNPE group. Insulin has multiple potential sites of modification, but literature precedent suggests that the site of modification will likely be the

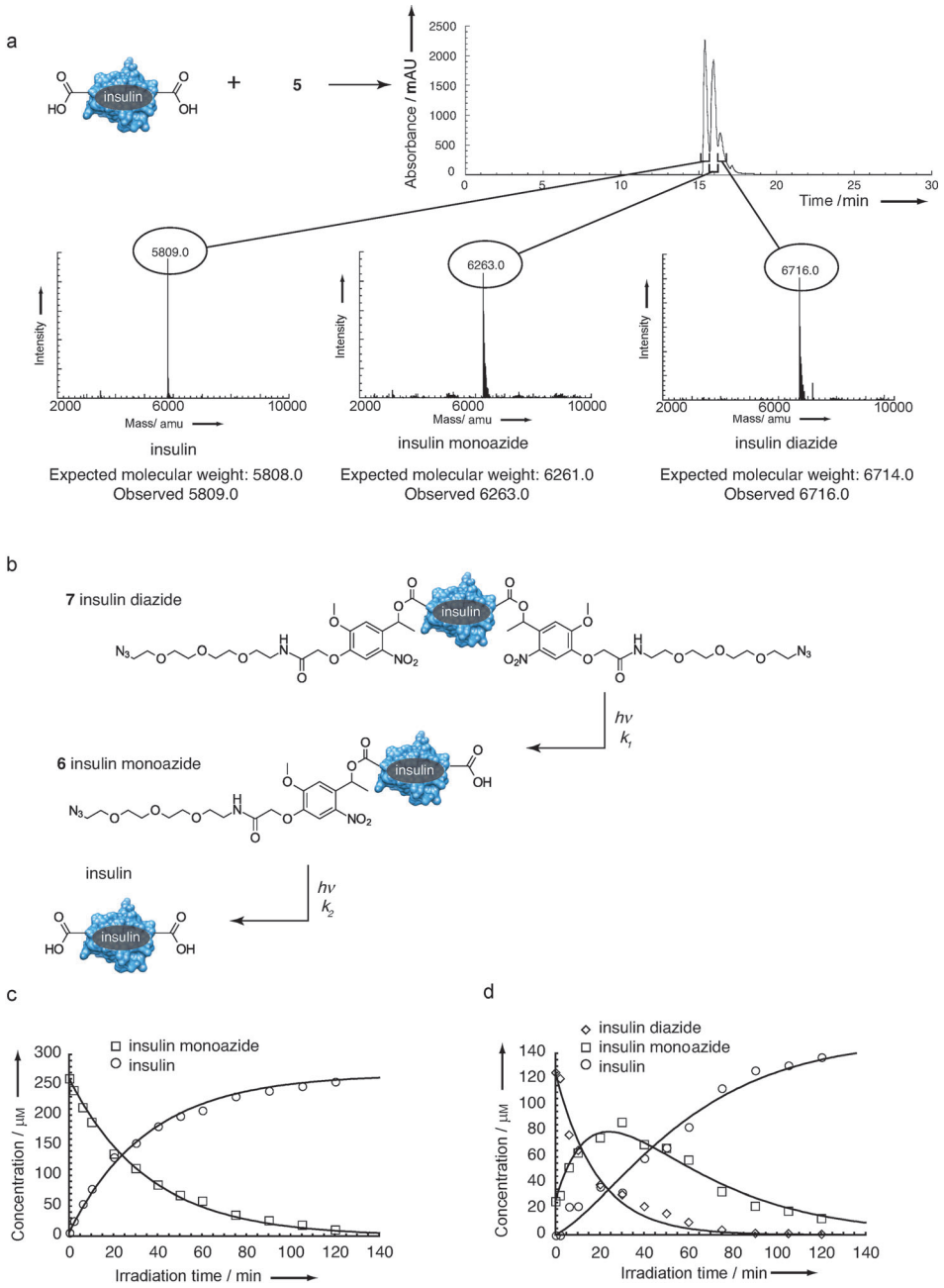

Figure 3. Formation and kinetic analysis of insulin monoazide and diazide photolysis. a) Reaction of insulin and compound **5** in a ratio of 1:2, resulting in the formation of insulin, insulin monoazide, and insulin diazide. HPLC analysis shows product ratio, and identity is confirmed by ESI–MS. b) Scheme showing the conversion of insulin diazide to insulin monoazide to insulin by photolysis. Rate constants indicated. c) Photolysis of insulin monoazide to insulin. First-order kinetic fit curves are shown. d) Photolysis of insulin diazide, first to insulin monoazide, then to insulin. Kinetic fit curves of two sequential first-order processes are shown.

carboxy groups. Because of this, insulin monoazide **6** will likely be a mixture of species with modifications at these different sites. Furthermore, insulin diazide **7** will be an even more complex mixture. It is possible that the micro environment of the modification site will influence the rate of photolysis, or alternatively, different sites could have essentially identical rates. By examining the kinetic fits of both the insulin monoazide and insulin diazide, we hoped to reveal these differences.

A solution (150 μL) of purified insulin monoazide (260.3 μM insulin monoazide **6**, 6.5 μM insulin) was irradiated for varying durations of time using a black-ray lamp (Model XX-15 L, 30 W). Samples were removed and analyzed by HPLC to quantify the amount of insulin and insulin monoazide. Both of these kinetic progress curves were plotted and fit well to a first-order process, giving a first-order rate constant k of $(0.028 \pm 0.001)\ min^{-1}$ (Figure 3c; also see the Supporting Information for details of fitting procedures).

In a parallel experiment, a purified solution (150 μL) of insulin diazide (125.2 μM insulin diazide, 26 μM insulin monoazide) was similarly photolyzed and analyzed. In this case, we determined the insulin diazide starting material, the insulin monoazide intermediate, and the final insulin as a function of time. The resulting kinetic plots were fit to two sequential first-order processes, corresponding to starting material being converted to intermediate, finally being converted to final product and resulting in two kinetic constants k_1 and k_2 as shown in Figure 3b.[7] The consumption of **7** (Figure 3d) was well fit with a first-order rate law, giving a first-order constant k_1 of $(0.054 \pm 0.004)\ min^{-1}$. This is essentially double the constant for the photolysis of insulin monoazide; this value is consistent with two DMNPE groups per molecule. The formation and consumption of **6** was well fit by two first-order rate constants, k_1 being $(0.052 \pm 0.004)\ min^{-1}$ and k_2 being $(0.023 \pm 0.001)\ min^{-1}$. Again, k_1 being approximately two times the value of k_2, and the first-order constant for the photolysis of insulin monoazide. The final step in the photolytic path, the formation of insulin, was less well fit by two first-order processes but gave comparable values of k_1 of $(0.028 \pm 0.017)\ min^{-1}$ and k_2 of $(0.033 \pm 0.019)\ min^{-1}$. Taken together,

the similarity of rate constants for the consumption of insulin monoazide and diazide, when corrected for the 2:1 ratio of photolyzed groups per mole of compound, suggests that the many possible sites of photolysis have essentially the same photoreactivity; thus, insulin monoazide, despite being a likely mixture, can be reasonably modeled as a monolithic entity with a single first-order photolysis rate.

In addition to photolysis by using a fluorescent UV lamp, we also examined rates of photolysis of insulin monoazide by using an LED (200 mW 365 nm LED, Nichia), since this is a light source closer to what might be used in a real-world application. A similar experiment was performed, in which the sample was placed close to the LED. Again, this was done to simulate a real-world situation, in which the light source was placed directly above the depot site. Sampling and analysis was performed identically to the manner described above. The resulting release was very rapid, giving a k_1 of $(0.893 \pm 0.105)\ min^{-1}$, approximately 32 times faster than the photolysis when using the fluorescent UV lamp (see the Supporting Information for details).

We then examined the photolysis kinetics of the resin-linked insulin by using the LED. This experiment was performed in a similar fashion to the solution-based experiments described above, with some key modifications: After each time period and before sampling, the resin was gently vortexed in an attempt to approximate homogeneity. In addition, the supernatant was sampled both directly after the LED was turned off, and then again after a dark period. This was done to insure that processes that were not based on photolysis were not taking place. The resulting "step-plot" is shown in Figure 4a. We consistently observed no additional release during the dark phase. The data in Figure 4a was replotted, with insulin release being plotted as a function of irradiation time. Even though the solution is inhomogenous and there were small variations in the total volume, the resulting curve was reasonably fit to a first-order rate law (Figure 4b). The resulting first-order constant ($k = (0.035 \pm 0.002)\ min^{-1}$) was approximately 25-fold slower than the result obtained for the homogeneous solution of insulin monoazide when using the same LED source. This is not that surprising, considering the possibility of scattering of the irradiation source by the beads, an effect that is not seen in the homogenous experiment.

In this work, we have prepared and characterized an insulin photoactivated depot (i-PAD), and in the process we have shown that insulin can be efficiently linked to an insoluble resin, maintain its integrity, and then be released in a predictable fashion by using light. As such, it forms an ideal starting point for the development of a therapeutic approach, in which a biodegradable photoactivated depot of insulin is injected under the skin and transdermal illumination stimulates the release of insulin at the appropriate time and in the appropriate amounts.

Insulin is particularly suited to this approach, since only a small amount is required for each day, and the moment-to-moment needs are highly variable. An insulin PAD would also put in place half of the components needed for an artificial pancreas, the other half being a continuous blood glucose monitor.[8] The combination of continuous blood glucose monitoring and continuous metered insulin administration should allow for native levels of blood sugar control and a return to normality for type I diabetics.

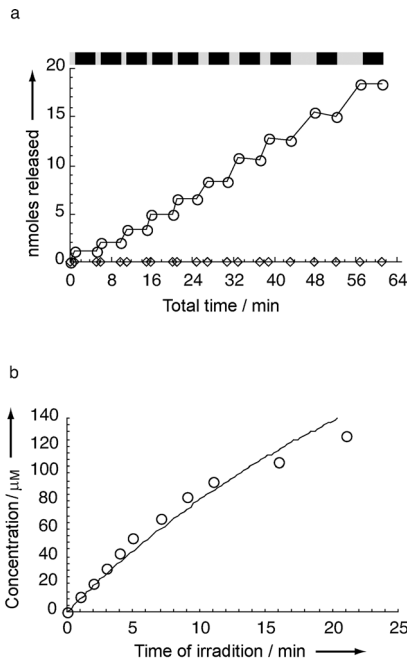

Figure 4. Stepwise photolysis of the photoactivated insulin depot. a) Cumulative moles of insulin released from the modified resin when using an LED point source that was turned on and off repeatedly. Light and dark bars indicate periods of irradiation and darkness. b) Replot of data from (a) shows the concentration of released insulin as a function of irradiation time. First-order fit curve is shown.

To our knowledge, this is the first example of light-controlled release of insulin from a covalently linked depot. There have been other descriptions of light-activated drug release in general. Many of them rely on degrading a bulk polymer by using light to allow release of the drug.[9] This process may be harder to control as there is no direct one-to-one correspondence of a photolysis event with drug release. In fact, photolysis events will be associated with random changes to the sequestering polymer, thereby making it difficult to predict changes in insulin release. Other examples from the literature use shorter and more toxic wavelengths of light and/or irreplaceable depots, for example, using 266 nm light to release fluorouracil from a difficult to replace intraocular lens.[10] A previously described approach of using photocleavable groups to make a drug insoluble (e.g. ibuprofen) would be difficult to adapt for insulin, because multiple modifications would be required, and this would again lead to challenges in predictable control of release.[11] Our approach of linking a single photolysis event to the release of a single drug should allow for a more predictable control of the process.

Future optimization steps include the use of a biodegradable resin, as well as the exploration of photocleavable groups that can be removed by applying longer wavelengths. This will allow depots to be positioned at deeper levels, including intramuscularly. However, the currently used photocleavable chromophore, DMNPE, may still be effective in shallow,

dermal or hypodermal sites, which are within approximately 1 mm of the skin's surface. The dermal layer is vascularized and adjacent to cells that synthesize vitamin D by using light of even shorter wavelengths (270–300 nm), which are able to penetrate to that depth. As such it may be an effective location for DMNPE-based depots.

The long-term benefit of such materials would be the transformation of the delivery of insulin and similar therapeutics: molecules that are needed in small and highly varying amounts in response to physiological cues.

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