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Hydrophobic Tags for Highly Efficient Light Activated Protein Release

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Abstract

We have previously described the Photoactivated Depot (PAD) approach for the light stimulated release of therapeutic proteins such as insulin. The aim of this method is to release of insulin from a shallow dermal depot in response to blood glucose information, using transcutaneous irradiation. Our first approach utilized a photocleavable group that linked insulin to an insoluble but injectable polymer bead. The bead conferred insolubility, insuring that the injected material stayed at the site of injection, until light cleaved the link, and allowed insulin to be absorbed systemically. While this proved to be effective, the use of a polymer to insure insolubility introduces two major design problems: 1) Low concentration of insulin, as a majority of the material is comprised of polymer, 2) Upon release of the insulin, the polymer has to be cleared from the system. To address these two problems, in this work we have pursued ‘hydrophobic tags’, photocleavable small non-polar molecules that confer insolubility to the modified insulin prior to irradiation without the bulk or need for biodegradation required of polymers. We developed a combined solid and solution phase synthetic approach that allowed us to incorporate a range of small non-polar moieties, including peptides, into the final depot materials. The resulting materials are >90% w/w insulin and have sharply decreased solubilities relative to unmodified insulin (1000x lower). We demonstrated that they can be milled into low micron sized particles that can be readily injected through a 31G needle. These suspensions can be prepared at an effective concentration of 20mM insulin, a concentration at which 120μl contains seven days of insulin for a typical adult. Finally, upon photolysis, the insoluble particles release soluble, native insulin in a predictable fashion. These combined properties make these new modified insulins nearly ideal as candidates for PAD materials.

Graphical Abstract

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Competing Financial Interests

The authors declare no competing financial interests.

Supporting Information

Complete and detailed materials and methods, including synthesis and characterization of intermediates and products. This includes HPLC and ESI-MS analysis.

Keywords

light control; insulin release; photocleavage; DMNPE; hydrophobic effect

Introduction

Recently we have pioneered the design and synthesis of light activated protein depots that release a specific protein in response to a light signal.¹ These have the potential to be useful for understanding and controlling a range of biomedically important processes. This work fits into a larger context of light controlled protein activity.^{2–14} Of particular interest to us is the control of insulin release from a shallow, dermal depot using an external light source (figure 1a). This light source can vary the amount of insulin released by varying the intensity and duration of the irradiation. The long term aim of such an approach, which we have called the Photoactivated Depot or PAD approach, is to allow an artificial pancreas without the use of an external pump and cannula. The controlled release of insulin in response to blood glucose has the potential to significantly improve the health outcomes for diabetics.^{15–22} We have demonstrated with first generation materials that the PAD approach works in-vivo, showing insulin release from the injected depot only after an external LED light source transcutaneously stimulates the material.²³ Furthermore we have shown we can deliver sufficient insulin in this manner to effect a robust reduction in blood glucose.

The first generation materials that we created, while showing efficacy, have multiple weaknesses that are linked to the design. A fundamental requirement of the PAD approach is that injected materials are insoluble and remain at the site of injection, until stimulated through the skin by an LED light source, at which point soluble, native insulin is released. We accomplished this with first generation materials by linking insulin using a light-cleaved linker to an insoluble but injectable polymer resin (figure 1b). In in-vivo experiments these particles were injected to form the depot. The use of a polymer resin to achieve insolubility however is responsible for several problems: 1) The insulin density of the material is low (<10% dry w/w), because of the high molecular weight of the polymer, limiting the potential length of time the depot can last. 2) Low insulin density also makes photolysis harder, because it is accompanied by low density of photocleavable groups. 3) After photolysis, a polymer remains and needs to be cleared. While clearing is possible via a biodegradable polymer, this introduces new design challenges, namely insuring that the polymer is stable until the insulin is consumed and then rapidly is cleared.

To avoid the problems associated with polymer based materials, we are seeking ways of toggling protein release that rely on the smallest possible modification to induce insolubility (e.g. < 1000 MW). We call this the 'tag' approach. The smallness of these modifications addresses all of the weaknesses of first generation materials: 1) The final materials can be as much as 90% dry w/w insulin, as the bulky polymer is replaced with a small tag. 2) After photolysis releases this tag, because it is a small molecule, it can be directly resorbed, and does not require the biodegradation of a polymer.

We have previously explored this approach with 'charge tags' that modulate insulin's pH dependent solubility by shifting the isoelectric point of the modified insulin.^{24, 25} In this work, we instead explore the use of 'hydrophobic tags', small non-polar moieties that we link to insulin via light cleaved linkers (figure 1c). These create modified insulins that are highly insoluble until irradiation releases native, soluble insulin. Using a combination of solid phase and solution phase synthesis, we have made hydrophobic tags that incorporate peptidic and unnatural elements. The variability of the peptidic elements has allowed us to systematically increase non-polarity, and make a family of modified light activated insulins.

With these we demonstrate that we can improve the pre-irradiation insolubility of the modified insulin by 94-fold over the previous charge-tag approaches. Furthermore, we show that these materials cleanly release native insulin in response to light, in a predictable fashion. Furthermore, we demonstrate that one of these materials, CD-insulin, can be made into an injectable suspension that contains 20mM insulin, a concentration which is consistent with 7 days of insulin therapy being contained in a 120 μ l injection.

Methods

Materials

Acetovanillone, tert-butylbromoacetate, 70% nitric acid, hydrazine monohydrate, manganese dioxide, Sigmacote, cyclododecylamine 97% and human recombinant insulin were purchased from Sigma Aldrich. Acetone, acetonitrile, isopropanol, dimethyl sulfoxide, N-methylpyrrolidone, diethyl ether, dichloromethane, methanol, ethanol, ethyl acetate, sodium chloride, magnesium sulfate, trifluoroacetic acid, 1N hydrochloric acid, acetic anhydride, sodium bicarbonate, 50% glutaraldehyde solution, Coomassie Brilliant Blue R-250 and Crocein Scarlet 3B were purchased from Fisher Scientific. Chemmatrix Rink amide resin was purchased from Protein Technologies. Piperidine, HATU, DIEA, Fmoc-Pro-OH, Fmoc-Val-OH, Fmoc-Ile-OH were purchased from Chem-Impex International Ltd. 10X IEF cathode buffer, 10X IEF anode buffer, IEF standards (pI 4.45–9.6) IEF sample loading buffer, pH 3–10 Criterion IEF gels were purchased from Bio-Rad. Peptide synthesizers and reaction vessels were siliconized by treating with Sigmacote for 5–10 minutes and, washed with acetone and dried before carrying out reactions on solid phase.

HPLC

All materials were analyzed by HPLC using a Nucleosil 150 $\times$ 3.2 mm 5 μ m C18 column (Supelco Analytical from Sigma Aldrich). Purification of modified insulin species was

performed either on a Microsorb-MV 250 × 4.6 mm 5 µm C18 column (Agilent), or a 250 × 21.2 mm 10 µm C18 column (Phenomenex).

Spectroscopy

The following instruments were used for spectroscopic analyses:

USB-2000 fiber optic spectrometer (Ocean Optics, Inc.) with DT-Mini-B lamp source for UV-visible absorbance spectroscopy, Agilent 1260 and 1050 systems with attached vacuum degasser, auto-autosampler and diode array detector for chromatography, ABI-Q-Trap 3200 for mass spectrometry, and Varian Inova 400 MHz NMR.

Injectability

Injectability assay was performed using a Hamilton 500 µL, Model 1750 TLLX SYR, Instrument Syringe (Catalog # 81222). 31G needle was also purchased from Hamilton (31 gauge, Metal Hub Needle, 2 in, point style 3, 6/PK; Catalog # 91031).

Synthesis

Detailed synthetic procedures, including HPLC and spectroscopic analysis of intermediates and products may be found in the supporting information section.

Solubility Studies

Solubility of all the hydrophobic insulins was determined as previously described.²⁴ Briefly, 100 nmol of insulin particles were suspended in 100 µl 10 mM PBS 7.2, in Eppendorf tubes. pH was adjusted as required. Tubes were shaken for 2 hours at 300 RPM. Tubes were then centrifuged (15000g, 5 minutes) and the amount of hydrophobic insulin in the clear supernatant was analyzed by HPLC. The pellet obtained after centrifugation was dissolved in DMSO to quantitate the amount undissolved and ensure that saturation was achieved.

Isoelectric Point Determination

Isoelectric points of modified insulins were determined by IEF gel electrophoresis as described previously.²⁴ IEF gels were run on a Bio-Rad Criterion vertical midi-format electrophoresis cell using Criterion pH 3–10 gels. Briefly, 1 nmol of hydrophobic insulin (8 µl) each were diluted to 10 µl with IEF sample buffer. Samples and IEF standards were loaded on the IEF gel. 1x cathode and anode buffers were added in cathode and anode chambers respectively. The gel was run for one hour at 100 V, another one hour at 250 V and an additional 30 minutes at 500 V. The gel was then fixed with 15% glutaraldehyde in 30% methanol for 2 hours. It was then washed with 27% isopropanol, 10% acetic acid solution. The gel was then stained for 45 minutes with a 0.1x staining solution (1x composition : 0.04% w/v Coomassie Blue R-250, 0.05% w/v Crocein Scarlet in 27% isopropanol, 10% acetic acid). Destaining of gel was performed with 40% methanol, 10% acetic acid solution. Images of the gel were captured and migration distance was analyzed on Adobe Photoshop to determine the isoelectric point.

Photolytic insulin release kinetics

Photolysis experiments were performed using a Nichia 200 mW 365 nm LED in 1 mL HPLC glass inserts (Fisherbrand, 03-391-23). Kinetics of insulin photorelease from hydrophobic insulins were determined as previously described.²⁴ Briefly, 100 nmol of insulin particles were suspended in 100 μ l 10 mM PBS 7.2, and transferred into HPLC glass inserts. The insert was placed 5 cm away from a 365 nm LED of known intensity. Materials were exposed to light for specific time intervals – samples at each time point were collected by centrifuging (15000g, 5 minutes) the tubes. A control tube containing 100 nmol was also used for each hydrophobic insulin, which is not exposed to light. The amount of insulin released into the supernatant was analyzed by IEF gel. Insulin was estimated using an insulin standard curve run on the same gel. Band intensity was determined by analyzing the pictures using Adobe Photoshop. Insulin release into the supernatant was plotted as a function of time and fitted using the first order equation, $\text{Insulin} = \text{Modified-insulin}_{t=0} [1 - \exp^{-kt}]$ in Kaliedagraph.

CD-insulin injectability

2 μ mol CD-insulin was suspended in 80 μ l 10 mM PBS pH 7.2. The pH of the suspension was adjusted with a minimal quantity of NaOH. The final volume of the suspension was adjusted to 100 μ l to achieve a concentration of 20 mM.

The material in the Eppendorf tube was milled with a motorized homogenizer for 4 minutes on ice. The tube was centrifuged at 1000g for 2 minutes to settle froth. Particles obtained as a pellet were re-suspended in the supernatant with the help of a pipettor, taking care that froth is not regenerated.

The injectability of this suspension was tested by loading it into a syringe with a 31G needle. The suspension was passed through the needle into a HPLC glass insert by pushing the plunger. The amount of CD-insulin that passed through the 31G needle was quantitated by diluting the suspension in DMSO and quantitated by UV spectroscopy (345 nm, using extinction coefficient $4470 \text{ M}^{-1} \text{ cm}^{-1}$). Additional PBS was used to wash the syringe and extract any traces of CD-insulin particles left in the syringe. This extra buffer was collected in another HPLC glass insert and quantitated by UV spectroscopy (345 nm), by diluting it in DMSO. This wash step was repeated until no more CD-insulin was seen in the buffer.

Results and Discussion

We synthesized two types of hydrophobic tag insulins: peptide and non-peptide based. The use of peptides permitted us to readily vary the non-polarity to allow the synthesis of families of modified insulins for ultimate testing. We synthesized three different non-polar tripeptides (sequences III, VVV, VPI). The terminal amine of the resin bound tripeptide was condensed with a ketone precursor to the DMNPE (dimethoxyl nitro phenyl ethyl) photocleavable group originally synthesized by Holmes (figure 2).²⁶ This ketone functionality was then converted on resin to the hydrazone. This step required significant optimization of solvent and reagent conditions to achieve robustness. For example, traditional polystyrene based resins did not work, but a crosslinked PEG resin (ChemMatrix)

did. Ultimately, we found that using a heated (65°C) suspension of 30mM resin-bound ketone, 3M hydrazine, and 300mM acetic acid in NMP/ethanol (90/10) quantitatively converted ketone into the hydrazone, independent of peptide sequence. These hydrazones were then cleaved from the resin using a 95/5 TFA/water cleavage solution. We did not observe reversion of the hydrazone into ketones despite the acidity of the cleavage solution. The resulting hydrazones were purified using reverse phase HPLC, and characterized by ESI-MS.

We faced another issue during the subsequent oxidation of these hydrazones to their corresponding diazo groups. We observed a significant amount of azine formation during this reaction, using our previously used conditions (165.6mM hydrazone, 4.6M manganese dioxide)²⁷ (See Supporting Information). This azine formation then interfered with the final ester formation between insulin and the reagent. Because azines can potentially be formed from a reaction of two hydrazones we hypothesized that the highly non-polar peptides may self associate during the reaction, driving the azine formation.^{28, 29} We therefore reduced the concentration of hydrazone during the oxidation reaction while maintaining the manganese dioxide concentration to preferentially favor the diazo formation over azine formation, and hence improve the yield of ester. Reducing the hydrazone concentration to 11 mM (a 15 fold reduction compared to our previous conditions) improved diazo and ester yields. The resultant diazo showed a distinct red color upon formation, with a corresponding 450nm absorption band. Due to its reactivity the diazo derivative was not further characterized, and was used directly. The reaction with insulin was performed with a 1:1 molar ratio of diazo to insulin (2 mM) in DMSO over a 24 hour period.

In addition to the three peptide based hydrophobic tagged insulins, we also synthesized a non-peptide based version, that incorporates the highly non-polar cyclododecyl amine (CD) group. The resulting “CD insulin” was synthesized in a similar fashion using the diazo precursor that we have previously described.²⁷ Including CD insulin, we then had four non-polar tag insulin materials (figure 3). We have previously shown that insulin that has been subjected to similar synthetic processing and photolysis retains bioactivity in-vivo.

The resulting products were purified using HPLC, and characterized by ESI-MS. Figure 4 depicts a representative HPLC and MS spectrum of one of these materials, CD-insulin, and the remaining are shown in Supporting Information. In the purified material, we see multiple closely clustered peaks, all with a similar 280nm/345nm ratio, that is characteristic of insulin with a single DMNPE modification. We expect this cluster, as there are six possible carboxyl groups in insulin, the site of attachment during the diazo reaction. We further characterized the three peptide tagged insulins using IEF gel analysis (Supporting information). These show the isoelectric point of insulin shifting from the native value of 5.4 to a more basic value of 5.9 for all three species III-insulin, VVV-insulin and VPI-insulin. This shift to the basic strongly supports that the materials contain carboxyl modified insulin, as these are the only acidic functionalities in insulin, the modification of which would shift the pI to the basic. Table 1 summarizes the MS and pI characterization for all four species.

Our first functional test of the hydrophobic tagged insulins was of insolubility. The main purpose of the hydrophobic tag is to lower the solubility of the modified insulin, until

photolysis cleaves the tag, and native, soluble insulin is released. Solubility was determined by adding 100 nmoles of each material to 100 μ l of 10mM pH 7.2 PBS. This was then shaken for 2 hours to allow equilibrium to be attained. The suspension was then centrifuged, and the supernatant sampled. HPLC was then used to quantify the amount of material that was dissolved in the buffer. The results were determined in triplicate and are summarized in figure 5. The solubility essentially follows the expectation based on a simple analysis of side chain non-polarity, with CD<III<VVV<VPI. All are highly insoluble compared to insulin with CD-insulin being 1300x more insoluble. CD-insulin is also 94x more insoluble than our previously described charge-tag material P2-insulin.²⁴

Insolubility is a necessary but not sufficient criteria for our materials to be effective PAD candidates. They also need to be injectable, and contain sufficient moles in a reasonable volume to last one or more days. The challenge is that the materials are insoluble by design. To allow them to be injected we used a small homogenizer to mill the solid material into smaller, and possibly injectable particles. We used a Zetasizer Nano to analyze the particle size distribution, and these results are summarized in table 2. They show that all four materials could be milled using this approach into particles with diameters that are in the low or sub-micron range, sizes that are well within the luminal diameter of a 31G needle. To confirm this, we created a 20mM suspension of the CD insulin, and expelled it through a 31G needle. The concentration of CD-insulin was determined before and after injection, and any retained CD-insulin was rinsed from the syringe and quantitated. Using this approach we observed <1% of the material was retained in the syringe (depicted visually in figure 6).

We then examined the ability for all four materials to release native soluble insulin in response to irradiation. 100 nmoles of a given material was suspended in 100 μ l of 10mM pH 7.2 PBS buffer and was irradiated at a distance of 5cm using an 365nm LED light source with an intensity of 1.95 mW/cm² at that distance. At defined time intervals, the sample was centrifuged to settle the particles, and the supernatant removed and assayed for insulin using IEF gel electrophoresis. The removed buffer was then replaced with fresh buffer, the particles resuspended and irradiation continued. We replace the buffer after each time point to simulate the replacement of tissue fluid that is continuously perfused and replaced, fed by the surrounding capillary bed. The cumulative insulin release was then plotted as a function of time. The data was well fit to a first order rate law. We fit both the rate constant k as well as the final concentration. A representative gel and progress curve is shown in figure 7, and the fitted parameters for all four materials summarized in table 3.

We observe that the maximum released concentration is always less than the expected based on the starting moles at the beginning of irradiation (approximately 60–80% of the expected). We hypothesize that this discrepancy may be due to released insulin interacting with remaining depot material and enhancing aggregation and insolubility. Two main trends are observed in the kinetic behavior of the materials: Firstly, the rate constant varies over a two fold range with more non-polar species releasing insulin more slowly. At the same time, the most non-polar and most slowly photolyzing species, CD-insulin shows the greatest proportion of the total insulin released. It is important to note that the rate of insulin release is likely a superposition of both photolysis kinetics as well as other kinetic processes, such as escape of soluble insulin from the particle. The process of photolysis of the suspension is

depicted visually in figure 8 for the representative material CD-insulin. It shows the dense suspension at time 0 progressively clarifying with increasing irradiation, consistent with our quantitative kinetic results.

Conclusions

In this work we have described the synthesis of the most efficient light activated protein materials to date. We have accomplished this by incorporating small hydrophobic tags that markedly shift the overall protein solubility in a highly atom-efficient manner. Because of the size of the tag, the final materials approach ideality with an insulin content of the dry material of 90–95% w/w. The synthetic approach we have developed is flexible, and allows for the continuous variation of solubility. We have shown that the materials readily release native soluble insulin in response to irradiation from an LED light source and that the rate and completeness of this release is influenced by the overall non-polarity of the tag. The ability to easily vary the materials' non-polarity may prove particularly useful as the in-vivo behavior of these materials is revealed. While the DMNPE group used in this work is cleaved by 360nm light, we are actively pursuing the application of the concepts described in this work using photocleavable groups that photolyze in the visible range. This has the potential to lower phototoxicity and increase the depth of light penetration into tissue.

In addition to high *dry* insulin density, we have shown that suspensions of the materials can be made at very high concentrations, as high as 20mM insulin. This is a concentration at which a 120µl injection will contain 7 days worth of insulin for the average adult. In addition, milled particles of the materials are highly injectable. The combination of these properties increases the potential utility of these materials as tools for controlling protein release, in particular for the continuously variable light-activated release of insulin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Polymer versus non-polar tag approach for PAD materials:

a) The overall PAD approach, showing irradiation of dermal depot material to release therapeutic protein. b) First generation PAD approach, utilizing a polymer linked to insulin via a light cleaved linker. Light stimulates insulin release from this insoluble material. c) The non-polar tag materials described in this work. Insolubility is conferred by a small non-polar moiety. Photolysis then removes this, releasing soluble native insulin.

Figure 2. Synthetic approach for peptide based non-polar tagged insulin.

Peptide constructed on solid phase and then modified with the nitro-keto acid intermediate.

This is then converted to the hydrazone, and cleaved from the resin. Finally, the hydrazone is converted to the diazo, which reacts with insulin to make the final material.

Figure 3. All four non-polar tag species.

CD insulin (cyclododecyl), III-insulin (isoleucine-isoleucine-isoleucine), VVV-insulin (valine-valine-valine), VPI-insulin (valine-proline-isoleucine).

Figure 4. Characterization of CD-insulin, representative non-polar tagged species.

HPLC and ESI-MS analysis of CD-insulin. HPLC shows traces at 280nm (blue) and 365nm (red). Different peaks are due to varying carboxyl group modifications on insulin, and possibly diastereomer formation. MS is a mass reconstruction from multiply charged ions. HPLC and MS data for remaining three species is found in supporting information.

Figure 5. Comparison of solubility between modified and unmodified insulin.

Bars indicate the average of three determinations, with standard deviations indicated. All modified insulins show solubilities lower than unmodified insulin, with a $P < .0001$ by ANOVA.

Figure 6. Pictorial demonstration of non-polar tagged insulin injectability through a 31G needle.
a) Material in syringe prior to injection. b) Material after injection into tube. c) Rinse buffer in syringe prior to injection. d) Rinse buffer after injection into tube.

Figure 7. Photolytic release of native soluble insulin from non-polar tag materials.

IEF gel showing insulin released into supernatant upon different periods of irradiation. Plot of cumulative effective molarity of insulin released as a function of time. First order rate equation fit shown.

Figure 8. Pictorial depiction of soluble insulin release from insoluble non-polar tagged insulin (CD-insulin).

Two samples, experimental and control prepared at 1mM. Experimental samples were irradiated for the indicated amount of time, while control samples were not.

Table 1.
Summary of characterization of all modified insulins.

Expected and observed molecular weights, as well as isoelectric point, as determined by IEF analysis.

Species	Expected mass (amu)	Observed mass (amu)	pI
Insulin	5808	5810	5.4
VPI-insulin	6369	6372	5.9
VVV-insulin	6357	6359	5.9
III-insulin	6399	6398	5.9
CD-insulin	6226	6227	-

Table 2.
Particle sizes for all modified insulins.

Particle size was determined in triplicate, and standard errors are indicated.

Species	Particle size (nm)
VPI-insulin	674 ± 130
VVV-insulin	457 ± 191
III-insulin	629 ± 11.2
CD-insulin	1540 ± 124

Table 3.
Kinetic rate constants for the photolytic release of insulin from all modified insulins.

Rate constants and final concentration values were determined by fitting progress curve to a first order rate law. The standard error for the kinetic rate constant is shown.

Species	Photolysis yield (%)	k (min ⁻¹)	Fit error
CD-insulin	83.4	0.095	0.006
VPI-insulin	63.5	0.19	0.029
VVV-insulin	61.5	0.17	0.009
III-insulin	63.8	0.13	0.008