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Visible light activated high density materials for controlled in-vivo insulin release

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Abstract

In this work we describe the synthesis, characterization and ultimate in-vivo assessment of second generation insulin photoactivated depot (PAD) materials. These are the first to use visible light to stimulate insulin release, and have in-vivo performance that is twenty eight fold improved relative to first generation materials. This improvement is due to two major factors linked to the utilized chemistry: 1) We have incorporated the coumarin photocleavable group, which increases the photo-release wavelength into the visible range, enhancing tissue penetration of the light and 2) Photo-toggling of insulin solubility is produced by linking three insulin molecules to a central bridge via light cleaved groups, and not by bonding to a large polymer. The resulting trimer is therefore highly dense (87% insulin dry w/w) but retains the insolubility required of the approach. Only after irradiation with visible light is native, soluble insulin released from the dermal depot. This high density increases the amount and ease of insulin release, as the density of photolytic groups is 10–20 fold higher than in polymer based first generation materials. We have synthesized new azide- terminated coumarin linkers that we react with the amine groups of insulin. Using mass spectrometry methods we identify the sites of reaction and purify individual isomers, which we demonstrate have in-vitro photolysis rates that are within a factor of two of each other. We then reacted these terminal azide groups with a tri-dentate strained alkyne linker. We show that the resulting insulin trimer is highly insoluble, but can be milled into injectable particles that release insulin only in response to light from a 406nm light source. Finally, we demonstrate that these materials have significantly improved in-vivo performance, releasing twenty eight fold more insulin on a per energy basis than first generation materials.

Graphical Abstract

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ASSOCIATED CONTENT

Supporting Information

(Complete and detailed materials and methods, including all assays, experimental procedures, synthetic methods and characterization of intermediates and products. This includes HPLC, ESI-MS analysis and NMR.

The Supporting Information is available free of charge on the ACS Publications website.

Keywords

insulin; coumarin; photolysis; click chemistry; protein chemistry

Introduction

We have previously shown that we can control insulin release from an injectable dermal PhotoActivated Depot (PAD) in diabetic animals using transcutaneous irradiation of the injection site.^{1–4} PAD materials are intended to be used with a small light source placed directly on top of the injection site. This then provides variable light stimulation in response to blood glucose measurements. In addition, the source can shadow the injection site to prevent ambient light from triggering insulin release. We have shown that the light-released insulin retains bioactivity and is able to effect blood glucose reduction. The purpose of such materials is to allow for the continuously variable insulin release that an insulin pump provides without the problems associated with pump use. These problems include cannula biofouling, crimping, snagging, and occlusion as well as infection.^{5–8} Other approaches for continuously variable insulin delivery include smart insulin or glucose-sensitive insulin delivery,^{9–10} red blood cells for insulin delivery¹¹, ultrasound-mediated delivery¹², oral insulin delivery¹³, NIR light mediated photothermal insulin release^{14–15} and microneedle based technologies¹⁶.

As effective as our first generation photoactivated depot material is, it has two major deficits associated with it. The first of these deficits is the use of a polymer to make the insulin insoluble, and thus stay at the site of injection. The polymer in our first materials makes up 90–95% of the final dry material weight, which limits the potential duration of the action for the material, as well as decreases the ease of photolysis. The higher the density of photocleavable groups, the higher the expected photolysis rate. The use of polymers to confer insolubility also necessitates the engineering of the polymer to be stable during the duration of depot use, but then be unstable and cleared after insulin consumption. This is a significant design challenge. A second major deficit of our first generation material is the use of the di-methoxy nitro phenyl ethyl (DMNPE) photocleavable group to link insulin to the polymer. The DMNPE group contains an ortho nitro benzyl group that is converted into a nitroso functionality during photolysis, which may lead to toxicity.^{17–18} In addition, the

DMNPE group requires 365nm UV light to cleave.^{19–20} Photocleavable groups that cleave in the visible range can potentially have less phototoxicity, and greater ease of photolysis, as longer wavelengths penetrate tissue more deeply. For example, Ash et al. suggest that the approximate skin penetration depth of light is 0.3mm for UV, 1mm for blue, 2.5mm for green and 4mm for yellow light.^{21–23}

To address both of these issues, in the current work, we have synthesized, characterized and ultimately analyzed in-vivo second generation materials that both eliminate the need for polymers to confer material insolubility, and incorporate coumarin photocleavable groups that cleave in the visible range (Figure 1).

To confer longer wavelength photo-release of the insulin, we have synthesized a new coumarin based photocleavable linker group based on the di-ethyl amino coumarin (DEACM) group that photo-cleaves at 406nm.^{24–25} In this new linker we have incorporated both an azide group and an alcohol group. The latter was linked to insulin amine groups via a carbamate linkage, producing two major isomers. We determined the sites of modification on the insulin by using a combination of protease cleavage and mass spectrometry analysis. These showed that the amino group of lysine was the major site of modification, and the N terminus of the A chain was the secondary modification site. Both of these isomers were shown to release native insulin upon photolysis. We further determined that the two major isomers photolyze at similar rates, and that these rates were well fit by first order kinetics.

To confer insulin insolubility without using polymers, we used the “trimer approach”. In this, insulin modified with azide-terminated coumarin groups, is reacted with a tri-alkyne central linker via a click reaction. We have previously found that this central linker is sufficient to confer insolubility to the final material. The result is that the final material is 87% insulin by dry weight. In a mixed aqueous/organic system in which the trimer was soluble, we observed during photolysis the transition to dimer and ultimately monomeric native insulin.

In order to use this highly insoluble material in animal studies, we had to make it injectable, and were able to accomplish this through milling it in a homogenizer into low micron sized particles, that were injectable through a 31G needle. We confirmed that these particles were ~2000 fold less soluble than native insulin. Finally, we demonstrated in diabetic animals that the material released insulin only in response to light, and that the released insulin retained bioactivity despite the extensive chemical manipulation required to synthesize it. Furthermore, we achieved a peak insulin level after a 30 second irradiation that represented a 5 fold increase in absolute insulin concentration, and a 28 fold increase in insulin release per applied light energy, relative to first generation, polymer based materials. This substantial and significant improvement in performance over first generation materials is a consequence of near perfect density and higher accessibility of longer wavelength light to the dermal depot. In addition, it achieves a performance that is nearing that needed for efficacy in humans.

Methods

Materials

1,3,5-cyclohexane tricarboxylic acid, bromoethane, 11-azido-3,6,9-trioxaundecan-1-amine, and human recombinant insulin (catalog 91077C) were purchased from Sigma Aldrich. Sodium iodide, N,N-diisopropylethylamine (DIPEA), *p*-xylene, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), dichloromethane (DCM), acetonitrile (ACN), methanol, ethanol, ethyl acetate (EtOAc), sodium chloride, trifluoroacetic acid (TFA), 1 N hydrochloric acid, 10 N sodium hydroxide, magnesium sulfate, methyl-4-bromobutyrate, selenium dioxide, sodium borohydride, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), carbonyldiimidazole (CDI) and 4-dimethylaminopyridine (DMAP), PBS (Gibco 10010023, KH_2PO_4 1 mM, NaCl 155.2 mM and Na_2HPO_4 3 mM), flat bottom glass vial inserts one mL, height 40 mm (catalog 03-391-23) and molecular sieves were purchased from Fisher Scientific. DMSO and DMF were stored in glass vials containing molecular sieves and used as stated. Additional reagents/components were: dibenzocyclooctyne amine (Click Chemistry Tools), HATU & 7-amino-4-methyl coumarin (Chem-Impex), hydroxybenzotriazole (HOBt) (Peptide International), Microvette 100 μL Li-HEP tubes for blood collection (Sarstedt), ultrasensitive Insulin ELISA kits (Alpco), MS grade trypsin (Pierce, PI90057), endoproteinase GluC (NEB, 50-811-915).

HPLC and spectrometry methods

UV-Vis analysis was performed using a USB-2000 fiber optic spectrometer (Ocean Optics, Inc.) with a DT-Mini-B lamp source. HPLC analysis was performed using modular Hewlett Packard Agilent 1050 and 1260 series instruments with an attached vacuum degasser, autosampler and diode array detector. C18 Hypersil (5 μm , 150×3.2 mm, Varian) columns were used as stated in the methods below. HPLC-MS was performed using a modular Agilent 1100 series with DAD coupled with a Q-Trap mass spectrometer (ABI) in a positive ion mode. For HPLC-MS analysis C18 Hypersil (5 μm , 150×3.2 mm, Varian) column was used. ESI-MS analysis was performed using a 3200 Q-Trap mass spectrometer in infusion mode.

Particle size measurements of insulin trimer

To 46.6 nmol of insulin trimer (or 140 nmol of caged insulin) which is dried in a tube, 150 μL of PBS was added. The pellet was ground by a motored pestle (Argos, A0001) for four minutes. It was centrifuged for one minute at 7000 rpm and the supernatant was removed. The settled particles were suspended in 50 μL PBS and 2 μL of the suspension was diluted to two mL of PBS to measure the particle size using DLS particle size analyzer (Zetasizer Nano ZS).

Solubility measurements of insulin and insulin trimer

The solubility limit was measured for coumarin insulin trimer and insulin in PBS to determine changes in insulin solubility. The extinction coefficient for insulin and caged insulin were determined in PBS and DMSO respectively. For trimer solubility measurements, to 23.3 nmol of insulin trimer (or 70 nmol of caged insulin) which is dried in

a tube, 150 μ L of PBS was added. The pellet was ground by a motored pestle (Argos, A0001) for four minutes. Additional 0.3 mL of PBS was used for washing the plastic probe and this volume was added to the original tube. The pH was adjusted to 7.2 and then they were centrifuged at 13000 rpm for three minutes. The supernatant was removed. The settled particles were suspended in 70 μ L PBS and shaken vigorously on Vortex-Genie 2 vortexer at speed 6 for two hours. After two hours, it was centrifuged for three minutes at 14000 rpm. The supernatant was removed, and its concentration was determined by UV-vis spectroscopy by diluting appropriately in DMSO. The measured extinction coefficient of CIMA at 380 nm in DMSO is 19770 $M^{-1}cm^{-1}$. Insulin solubility was measured in the same manner. Insulin (10 mg, 1.72 μ mol) was mixed with 0.2 mL of PBS in three tubes for triplicates. The pH of these solutions was adjusted to 7.2. These tubes were vortexed and centrifuged in an identical manner as mentioned above. Insulin concentration was measured using UV-vis spectroscopy by diluting appropriately in PBS. The measured extinction coefficient of insulin at 280 nm in PBS is 5233 $M^{-1}cm^{-1}$.

Continuous irradiation of insulin trimer in PBS for rate constant determination

For this photolysis, to 46.6 nmol of insulin trimer (or 140 nmol of caged insulin) which is dried in a tube, 150 μ L of PBS was added. The pellet was ground by a motored pestle (Argos, A0001) for four minutes. Additional 0.3 mL of PBS was used for washing the plastic probe and this volume was added to the original tube. The pH was adjusted to 7.2 and then solutions were transferred to small glass vials (Fisher Scientific, catalog # 03-391-23) in which the photolysis was performed. These vials were centrifuged at 7000 rpm for one minute. The supernatant was removed. Before initiating the photolysis, the settled particles were suspended in 100 μ L PBS, vortexed at high speed briefly and centrifuged at 7000 rpm for a minute. The supernatant was removed and labeled as $t=0$. Another 100 μ L PBS was added, the sample was vortexed vigorously and photolysis was performed at this point and the samples were irradiated for 32 minutes total under 406 nm LED at 6.4 cm distance. At each time point, the irradiation was stopped, the sample was vortexed and centrifuged for one minute at 7000 rpm. Then the supernatant was removed and replaced with the same volume of fresh PBS. For gel analysis, 5 μ L of collected supernatant was mixed with 5 μ L Laemmli 2x gel loading buffer and ran on the gel. The bands from the gel were analyzed using Photoshop and cumulative insulin released was calculated. The data was fit in Kaleidagraph software using the following equation: $y=A_0(1-e^{-kt})$ where A_0 is the starting concentration.

Photolysis of insulin trimer in DMSO

For performing this photolysis, 1.33 nmol of the trimer (or 4 nmol of CIMA in the form of trimer) were added to a solution of 20% H_2O in DMSO at a concentration of 0.08 mM. The solution was transferred to a 1.7 mL microfuge tube in which the photolysis was performed by keeping it at 6.4 cm distance from 406 nm LED. At each time point, the photolysis was paused, the solution was vortexed and 5 μ L of it was taken. For the final analysis, this 5 μ L was mixed with 5 μ L of 2x Laemmli buffer and ran on the gel. The bands were quantified by Photoshop and amount of insulin corresponding to each band was calculated using an insulin standard curve which was run and analyzed in the same manner. The photolysis was performed in triplicates.

Discrete irradiation experiment of insulin trimer

For this photolysis, to 46.6 nmol of insulin trimer (or 140 nmol of caged insulin) which is dried in a tube, 150 μ L of PBS was added. The pellet was ground by a motored pestle (Argos, A0001) for four minutes. Additional 0.3 mL of PBS was used for washing the plastic probe and this volume was added to the original tube. The pH was adjusted to 7.2 and the solutions were transferred to small glass vials (Fisher Scientific, catalog # 03-391-23) in which the photolysis was performed. These vials were centrifuged at 7000 rpm for one minute. The supernatant was removed and saved. Before initiating the photolysis, the settled particles were suspended in 100 μ L PBS, vortexed at high speed briefly and centrifuged at 7000 rpm for a minute. The supernatant was removed and labeled as t=0. Another 100 μ L PBS was added and photolysis was performed at this point and the samples were irradiated for two minutes under a 406 nm at 6.4 cm distance. The supernatant was collected again after vortexing and centrifuging. All time points were collected in this manner. Another brief two minutes irradiation was performed at t=65 minutes. The control samples were treated in an identical manner except for the light from LED was blocked by placing an aluminum foil between LED and glass vial. For gel analysis, 5 μ L of collected supernatant was mixed with 5 μ L Laemmli 2x gel loading buffer and ran on the gel. The bands from the gel were analyzed using Photoshop and cumulative insulin released was calculated.

Thermal measurements on the skin

A scaffold containing 7 mm diameters holes with sets of magnets (for the purpose of LED alignment) was glued on rat skin after shaving. The magnets were placed in such a way that 406 nm LED could be centered on holes for irradiating specific targeted area on the skin. To measure the increase in temperature after irradiation, the skin area exposed through holes was irradiated for specified durations of time. Light source was removed post-irradiation, and pictures of the irradiated spot were taken immediately using FLIR Pro One infrared imaging camera. The pictures were then processed in FLIR Tools.

Absolute irradiance measurements of 406 nm LED

Absolute irradiance measurements were performed on a USB-2000 fiber optic spectrometer (Ocean Optics, Inc.) attached to a CC-3-UV-S cosine corrector (Ocean Optics, Inc.). In Spectrasuite software, absolute irradiance of 406 nm LED was recorded by shining the LED by positioning it in line with the cosine corrector at varying distances. Absolute irradiance values were plotted against inverse distance squared values.

In-vivo studies

Animals

Sprague-Dawley male rats were obtained from Charles Rivers Laboratories (Roanoke, IL). Diabetes was induced by treatment with 65 mg/kg Streptozotocin (Sigma-Aldrich, St. Louis, MO). Diabetes was defined as blood glucose concentrations of >250 mg/dL on three consecutive days. The values were measured using new Freestyle glucometers and blood was obtained from the tail vein.

The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, eighth edition. The protocol was approved by the University of Missouri Kansas City Institutional Animal Care Use Committee protocol #1603.

Injection of PAD material

Rats were anesthetized with isoflurane gas using a precision vaporizer. The upper backs of rats were shaved prior to injection of the PAD materials. Injections of ~65–70 μ L of PAD material were made using a 1/2 cc syringe and a 27-gauge needle. The compact LED light source was anchored to the skin over the injection sites by two small dots of superglue. Rats' body temperature and hydration were maintained throughout the experiment.

Insulin analysis by ELISA

Blood samples were collected in Microvette 100 μ L Li-HEP tubes (Sarstedt) from the tail-vein using a glass capillary and stored on ice. After collecting all time points from an experiment, the samples were centrifuged at 5000 rpm for two minutes. The supernatant was removed and stored at -20°C until the ELISA analysis was performed. The ultrasensitive human insulin ELISA kit (Alpco, Salem, NH) was used according to the manufacturer's instructions.

Glucose measurements

Glucose measurements were made using brand new Freestyle glucometer and strips (Abbott, Alameda, CA) using plasma from blood samples obtained from the tail vein.

Statistical Analysis

The two-sample unequal variance t-test was used to calculate p-value between the experimental and control groups (Excel).

Results and Discussion

We previously have synthesized insulin photoactivated depot (PAD) materials by first linking a DMNPE photocleavable (PC) group to insulin via the reaction of a linker-bound diazo group and a carboxyl group on the insulin.¹ Our intention in this work was to install a coumarin PC group onto the insulin to increase the photocleavage wavelength into the visible light region. We originally explored a diazo linkage strategy, but found that forming a carbamate bond was simpler and more robust. The specific coumarin that we used was the DEACM group, that has been shown to photolyze efficiently in the visible range, i.e. $> 400\text{nm}$. Coumarins are a particularly well explored family of photocleavable groups, and manipulation of the structure allows extension of photolysis wavelength well into the visible range.^{26–27} Starting from the commercially available amino coumarin 1, we first installed an ester functionality through alkylation with methyl-4-bromo butyrate (Figure 2). We then alkylated the aromatic amine a second time with ethyl bromide to form compound 2. We observed that if we did not do a second alkylation, the subsequent oxidation step did not proceed cleanly, and furthermore, the coumarin light absorption was blue shifted. After alkylation, we oxidized the methyl group to the aldehyde using selenium dioxide, followed

by sodium borohydride reduction to the corresponding alcohol (compound 3). We then hydrolyzed the ester using hydrochloric acid to reveal the carboxylic acid synthetic handle of compound 4. This carboxylic acid was condensed with an azido-ether amine to make compound 5.

To attach insulin to this linker, we first activated the alcohol group using carbonyl-diimidazole to make the acyl-imidazole intermediate, and then reacted this with insulin to form compound 7, what we call “coumarin insulin mono-azide” or CIMA. Our expectation was that the amine groups of insulin would be the most likely to react with this species. Given that insulin has three primary amines (from the sole lysine side chain, and the N-terminal of the two subunits), we expect at the maximum three isomers. The crude HPLC trace following this reaction showed two major products. Each of these products was purified, and shown to be the singly reacted species, with a mass consistent with the carbamate product (Figure 3). With our first generation DMNPE based materials, we identify on HPLC many of the possible 6 isomers that result from the reaction of the diazo group with the insulin carboxyl groups. Here, we were interested in determining which amines on insulin were responsible for the two observed isomers, and, ultimately, if either of these photolyzed at an appreciably different rate from the other. To characterize the site of modification, we used a combination of protease cleavage and mass spectrometry.

We initially treated both isomer 1 and 2 with trypsin, which is expected to cleave on the carboxyl side of basic amino acids (arg and lys). The expected locations of cleavage and of CIMA modification are shown in figure 4. We anticipate a large species that contains both N termini. In addition we expect a smaller species that contains the lysine. Finally, we also expect the terminal threonine in chain B to be removed. With isomer 1, we isolated two main species: 1) A species with a mass consistent with the large fragment containing a single modification, and 2) A species with a mass consistent with the unmodified small fragment and showing an unmodified mass. This pattern indicated that one of the N-termini in isomer 1 was modified, but not which one (see Supporting information).

With trypsin treatment of isomer 2 (Figure 5) we isolated three main products: 1) A species with a mass consistent with the large fragment containing no modifications (figure 5a), 2) A species with a mass consistent with native insulin with a single CIMA group attached (Figure 5b) and 3) A species with a mass consistent with the small fragment singly modified (Figure 5c). This is consistent with a modified lysine side chain on isomer 2. We failed to observe the product that would result from the cleavage of the final threonine that follows the lysine on chain B. Modification of the lysine side chain is expected to abrogate trypsin cleavage due to elimination of the cationic side chain by acetylation. This further supports the lysine as the site of modification on isomer 2.

Because trypsin treatment of isomer 1 did not unambiguously indicate which of the N termini were modified, we further analyzed isomer 1 by using the protease GluC, which cleaves on the carboxyl side of glutamic acid residues (Figure 6). From this reaction, we isolated three of the possible four different species. Each had a mass that was consistent with the expected glutamic acid cleavage site on the native protein, except for the N terminal tetrapeptide on the A chain (sequence GIVE). This showed a mass consistent with the native

sequence modified with a carbamate linkage to the coumarin linker. As a final confirmation, this modified GIVE peptide was irradiated with a 406nm source, which regenerated the expected native GIVE mass. Thus, the combined protease/MS analysis confirmed the identities of the two isolated isomers.

We designated this coumarin modified insulin “CIMA” for Coumarin Insulin Mono-Azide, indicating that there was one azide-terminated linker attached to the insulin. We examined its ability to release insulin upon photolysis in a mixed DMSO/buffer (PBS) system (15% DMSO in PBS), by using a 406nm LED light source with an absolute irradiance of $2.32\text{mW}/\text{cm}^2$ at the irradiation distance of 6.4cm. Insulin release as a function of irradiation time was analyzed using HPLC (Figure 7). Individual purified isomer 1 and 2 were used for the study which allowed us to quantitate the rate of photolysis for each isomer. The data was well fit to a first order process, with isomer 1 giving a rate of 0.0096 sec^{-1} and isomer 2 giving a rate of 0.018 sec^{-1} . This two fold difference in photolysis rate may be due to differential solvent access, with the amine of the lysine being further separated from the bulk of the insulin, and thus more accessible to solvent. Photolysis of coumarins involves a solvolysis step of a photogenerated cation. Based on the similarity in photolysis rates, we used the mixture of isomers 1 and 2 to prepare trimer materials for animal studies.

Our next step was to incorporate CIMA into the final trimer material, via a click reaction of its azide functionality with a tris-DBCO linker that we have previously described (Figure 8)³. The reaction was performed overnight using the mixture of isomer 1 and 2, and the product purified by size exclusion chromatography. This yielded a high molecular weight species that we isolated, and confirmed was the desired trimer via ESI-MS (Figure 9). In PBS buffer, the trimer proved to be highly insoluble, as we had previously observed with related DMNPE materials³. This posed a challenge for animal studies, as it is necessary to inject the material to form the depot. To make the insoluble solid injectable, we mechanically milled it using a small homogenizer. This resulted in particles that had diameters in the low micron range, and were efficiently injectable, even through narrow 31G needles. The absolute solubility of this material in PBS buffer was analyzed in triplicate and compared to insulin, and was found to be >2000 fold less soluble ($1.0\mu\text{M}$ 0.22 se for the trimer, versus 2.1mM 0.18 se for insulin) (Figure 10). This is qualitatively similar to what we originally observed with the DMNPE based trimer material.

We confirmed that this trimer material could be photolyzed into insulin, by irradiating for varying times using the 406nm source in 1:4 H₂O:DMSO, and analyzing the products via polyacrylamide gel electrophoresis (Figure 11). Prior to irradiation, the trimer standard shows a gel mobility that is lower than insulin, consistent with its higher molecular weight. Upon photolysis, two additional bands form, the lower of which is consistent with insulin, and the middle, half way between the trimer and insulin is likely the dimer. The identity of the low band was confirmed by ESI-MS to be insulin (5809.0 MW observed, 5808.0 expected). The trimer and insulin appeared to stain with different intensities, which made fitting of this data to two sequential first order reactions difficult. The photolysis was also examined under aqueous conditions (PBS), a condition in which the material is a heterogeneous suspension. We irradiated a suspension of the trimer particles continuously, centrifuging and analyzing the supernatant for insulin presence at specific time intervals.

The buffer was replaced, the sample was then resuspended, and photolysis continued. The observed rate of release is shown in figure 12, and is again well fit by a first order equation.

Our final method of analysis was using a discrete irradiation of the trimer suspension for 2 minutes, followed by an analysis of released insulin in the supernatant. The intention behind this analysis is to observe the material's behavior in-vitro under conditions similar to in-vivo use, specifically the brief irradiation of the material to allow a dose of insulin to be delivered. After photolysis we observed a rapid release of two thirds of the insulin dose into the supernatant in the first five minutes, followed by a slower leveling off in which approximately a third of the total released moles was released in the next hour (Figure 13). We previously have observed this phenomenon in first generation polymer based PAD materials. This may be due to sub-pools of insulin in the particles that have greater and lesser accessibility to the bulk solvent. After a second irradiation of this material, we again saw a spike in insulin release, followed by a leveling off, although the total amount released was less than that in the first photolysis. It is unlikely that this slowing is due to consumption of insulin after the first irradiation, as only 14.3 % of the total loading is released in the first irradiation. Instead, it is possible that released insulin is trapped, through non-covalent association (for example as driven by dimerization or hexamerization onto still particle bound trimers of insulin).

Based on our observation of visible light stimulated insulin from our trimer material, we prepared for in-vivo studies. We have previously observed that irradiations with a light source that has too high a power can cause redness or even a burn days after the irradiation. Therefore we examined the effect of the 406nm light source on both acute skin temperature as well as redness over 48 hours as a function of irradiation time and attempted to correlate the two.

The power of the light source was modulated using spacers that increase the distance between the LED and the skin surface. We determined the absolute irradiance of the light source as a function of distance (in W/cm^2), confirming that it followed the inverse square law. Using this we then determined the absolute irradiance of the source in two configurations using two different size spacers. The two configurations gave skin absolute irradiance values of $0.11\text{W}/\text{cm}^2$ and $0.24\text{W}/\text{cm}^2$. We irradiated the skin with a series of regularly spaced circular spots with an area of 0.39 cm^2 using a template (Figure 14a). The duration of exposure was varied from 0 to 8 minutes. After each irradiation, we removed the source and immediately took a picture of the skin with a thermal imaging camera, which allowed us to determine the maximum and average skin temperature in the irradiation area (Figure 14b).

As expected, longer durations of exposure and higher source power resulted in higher temperatures on the skin within limits (Figures 14c). At the higher light intensity, we observed higher temperature with longer exposure times, and a burn forming 24 hours after the irradiation. However, the lower intensity light source showed a plateaued temperature at $\sim 45^\circ\text{C}$ and no skin injury (Figure 14d). It is interesting to note that the lower intensity source at the longest irradiation time (8 minute) delivers more energy to the skin than does the higher intensity source for a briefer amount of time, but the latter produces higher

temperature increases and consequent damage and the former remains cooler and undamaged. We interpret this as a heat flow issue, with the skin and surrounding tissue having a limited kinetic capacity to absorb energy. Any energy flux above a critical level will cause increased heating and subsequent tissue damage. Empirically, we have found that if the skin temperature is maintained at 45°C or lower, no damage is observed. We confirmed this by doing histology on the 0 and 8 minute time points, confirming similar overall structure. Based on these results, all of our in-vivo analysis of the trimer material was performed using the 0.11W/cm² light source configuration.

The in-vivo analysis was performed in a similar fashion as previously described⁴. Briefly, we injected insulin trimer material containing 140nmoles of insulin suspended in 50 µl of sterile PBS buffer (total final volume ~65µl) into the dermal layer of diabetic streptozotocin treated Sprague Dawley rats. A light source was positioned onto the injection site, and affixed using surgical adhesive. Control animals were treated identically, except the light from the source was blocked by a sheet of aluminum foil. Thus, any potential leaching, or effects of light source heat could be controlled for. We analyzed both blood insulin using an ELISA assay, and blood glucose using test strips and a glucometer. The experiment was performed with four experimental and four control animals. We determined in preliminary work (data not shown) that an irradiation of only 30 seconds was necessary. In this experiment, we used two separate 30 second irradiations, the first at time 0 and the second at 65 minutes. The purpose of this was to determine if we could increase insulin release with increased light, as we had previously observed with first generation materials⁴. The results are shown in figure 15.

We observe essentially no detectable insulin released in control animals, with blood insulin levels at baseline. This confirms that minimal insulin is leached in the absence of irradiation. Upon irradiation, experimental animals showed insulin release at the earliest time point (5 minutes), further supporting PAD released insulin as among the fastest insulins. In addition we observe an increase in insulin to ~0.68 nM at 65 minutes, a level approximately five fold higher than with our first generation materials (which peaked at 0.13nM during the first hour). In addition, upon a second 30' irradiation at 60 minutes, the insulin level rises higher to ~1.4nM. We didn't observe a peak in the insulin level. This may be related to the in-vitro observation of a second slower kinetic release process present in this material. This may extend the release in-vivo as well, resulting in the observed trend. Blood glucose significantly falls in experimental animals, with an ~75% reduction observed at 125 minutes, a level considered in the normal range. This is a significantly greater level of blood glucose reduction compared to our first generation materials, which showed a reduction in blood glucose after 125 minutes of 36%. The doubling we observe is consistent with the higher amount of insulin released with the coumarin trimer. Blood glucose reduction did achieve statistical significance (i.e P<0.05 for each time point), however blood insulin increase did not, likely due to the smaller number of animals used, and the noise inherent to animal data.

Conclusions

In this work, we have described the synthesis of new, highly effective depot materials for the light stimulated release of insulin in-vivo. By using a coumarin derivative to form the

photocleavable bond with insulin, we have succeeded in moving the release wavelength into the visible range. This has the effect of reducing the chances for phototoxicity, and increasing the amount of light that penetrates the skin. By using a tri-dentate linker, we were able to make highly insoluble materials, that were nonetheless injectable, once milled into low micron sized particles. These particles are 87 % insulin dry w/w %, making them approximately ten times more dense than first generation, polymer-based materials. Furthermore, they released native, soluble insulin in response to irradiation by a 406nm LED. We observed that this LED based light source could cause skin heating, but if the intensity was lowered by a factor of 9, skin temperature increases plateaued at 45°C, where no skin damaged is observed.

These new materials, in addition to having superior characteristics compared to first generation materials (higher density, longer wavelength release), also showed superior performance in-vivo. Despite having a shorter period of irradiation (30 seconds vs 120 seconds), we observed a 5.23 fold increase in peak insulin blood concentration, relative to first generation materials. This improved performance is even greater when normalized to the total amount of light energy applied during the material stimulation. In our studies of first generation polymer-based materials, we used a 365nm source with an intensity of 0.71 W/ cm². This irradiated a spot with an area of .385 cm² for 120 seconds. The result of this was a total applied energy of 32.8 J. With the coumarin based materials examined in this work, we irradiated with a 406nm source with an intensity of 0.11 W/ cm². This irradiated a spot with an area of 1.87 cm² for 30 seconds, leading to a total applied energy of 6.22 J. Thus, we released 5.23x the peak moles of insulin for 6.22/32.8 the energy, or a total efficiency improvement of 28 fold.

This higher efficiency of release is likely due to a combination of longer wavelength, which increases the amount of light reaching the depot, and higher density, which increases the rate of photolysis (due to higher concentration of photocleavable groups). With these materials we can release quantities of insulin that achieve blood concentrations that can easily exceed what is needed for a diabetic rat. The final step required for human application is to increase the total *moles* released, as humans have ~200 fold greater blood volume, and thus require greater number of moles. The intention behind the PAD approach is to have a single injection last for multiple irradiations over a day with the potential for multi-day use. In the trials described in this work, depots contained 140nmole of insulin, or approximately half of the daily requirement for a human adult. We estimate we are within a factor of 10 of the mole requirement for multi-day human application, which should be eminently achievable through a further exploration of the factors described in this work.”

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

DMNPE	di-methoxy nitro phenyl ethyl
DEACM	diethyl amino coumarin methyl
CIMA	coumarin insulin mono azide

REFERENCES

1. Jain PK; Karunakaran D; Friedman SH, Construction of a photoactivated insulin depot. *Angewandte Chemie International Edition* 2013, 52 (5), 1404–1409. [PubMed: 23208858]
2. Nadendla K; Friedman SH, Light Control of Protein Solubility Through Isoelectric Point Modulation. *J. Am. Chem. Soc* 2017, 139 (49), 17861–17869. [PubMed: 29192764]
3. Sarode BR; Jain PK; Friedman SH, Polymerizing Insulin with Photocleavable Linkers to Make Light-Sensitive Macropolymer Depot Materials. *Macromolecular bioscience* 2016, 16 (8), 1138–1146. [PubMed: 27171861]
4. Sarode BR; Kover K; Tong PY; Zhang C; Friedman SH, Light control of insulin release and blood glucose using an injectable photoactivated depot. *Molecular pharmaceutics* 2016, 13 (11), 3835–3841. [PubMed: 27653828]
5. Heinemann L; Krinelke L, Insulin infusion set: The Achilles heel of continuous subcutaneous insulin infusion. *J. Diabetes Sci. Technol* 2012, 6 (4), 954–964. [PubMed: 22920824]
6. Klonoff DC; Freckmann G; Heinemann L, Insulin Pump Occlusions: For Patients Who Have Been Around the (Infusion) Block. *J. Diabetes Sci. Technol* 2017, 11 (3), 451–454. [PubMed: 28355924]
7. Lenhard MJ; Reeves GD, Continuous Subcutaneous Insulin Infusion: A Comprehensive Review of Insulin Pump Therapy. *Arch. Intern. Med* 2001, 161 (19), 2293. [PubMed: 11606144]
8. Moser C; Maurer K; Binder E; Meraner D; Steichen E; Abt D; Freund-Unsinn K; Hofer SE, Needle detachment in a slim and physically active child with insulin pump treatment. *Pediatric Diabetes* 2015, 17(5), 385–388. [PubMed: 26201949]
9. Gu Z; Aimetti AA; Wang Q; Dang TT; Zhang Y; Veisheh O; Cheng H; Langer RS; Anderson DG, Injectable nano-network for glucose-mediated insulin delivery. *ACS Nano* 2013, 7 (5), 4194–4201. [PubMed: 23638642]
10. Hu X; Yu J; Qian C; Lu Y; Kahkoska AR; Xie Z; Jing X; Buse JB; Gu Z, H₂O₂-Responsive Vesicles Integrated with Transcutaneous Patches for Glucose-Mediated Insulin Delivery. *ACS Nano* 2017, 11 (1), 613–620. [PubMed: 28051306]
11. Wang C; Ye Y; Sun W; Yu J; Wang J; Lawrence DS; Buse JB; Gu Z, Red Blood Cells for Glucose-Responsive Insulin Delivery. *Adv. Mater* 2017, 29 (18).
12. Di J; Yu J; Wang Q; Yao S; Suo D; Ye Y; Pless M; Zhu Y; Jing Y; Gu Z, Ultrasound-triggered noninvasive regulation of blood glucose levels using microgels integrated with insulin nanocapsules. *Nano Res.* 2017, 10 (4), 1393–1402.
13. Banerjee A; Ibsen K; Brown T; Chen R; Agatemor C; Mitragotri S, Ionic liquids for oral insulin delivery. *Proc. Natl. Acad. Sci. U.S.A* 2018, 115 (28), 7296–7301. [PubMed: 29941553]
14. Teodorescu F; Oz Y; Qu C; Abderrahmani A; Foulon C; Lecoeur M; Sanyal R; Sanyal A; Boukherroub R; Szunerits S, Photothermally triggered on-demand insulin release from reduced graphene oxide modified hydrogels. *J. Control. Release* 2017, 246, 164–173. [PubMed: 27984105]
15. Timko BP; Arruebo M; Shankarappa SA; McAlvin JB; Okonkwo OS; Mizrahi B; Stefanescu CF; Gomez L; Zhu J; Zhu A, Near-infrared-actuated devices for remotely controlled drug delivery. *Proceedings of the National Academy of Sciences* 2014, 111 (4), 1349–1354.

16. Jin X; Zhu DD; Chen BZ; Ashfaq M; Guo XD, Insulin delivery systems combined with microneedle technology. *Adv. Drug Deliv. Rev* 2018, 127, 119–137. [PubMed: 29604374]
17. Firoze Khan M, G. A. S. A., Contribution of nitrosobenzene to splenic toxicity of aniline. *J. Toxicol. Environ. Health* 2000, 60 (4), 263–273.
18. Bruning-Fann CS; Kaneene JB, The effects of nitrate, nitrite, and N-nitroso compounds on animal health. *Vet. Hum. Toxicol* 1993, 35 (3), 237–253. [PubMed: 8351799]
19. Holmes CP; Jones DG, Reagents for Combinatorial Organic Synthesis: Development of a New o-Nitrobenzyl Photolabile Linker for Solid Phase Synthesis. *J. Org. Chem* 1995, 60 (8), 2318–2319.
20. Patchornik A; Amit B; Woodward RB, Photosensitive protecting groups. *J. Am. Chem. Soc* 1970, 92 (21), 6333–6335.
21. Ash C; Dubec M; Donne K; Bashford T, Effect of wavelength and beam width on penetration in light-tissue interaction using computational methods. *Lasers Med. Sci* 2017, 32 (8), 1909–1918. [PubMed: 28900751]
22. Anderson RR; Parrish JA, The optics of human skin. *J. Invest. Dermatol* 1981, 77 (1), 13–19. [PubMed: 7252245]
23. Hardy JD; Hammel HT; Murgatroyd D, Spectral Transmittance and Reflectance of Excised Human Skin. *J. Appl. Physiol* 1956, 9 (2), 257–264. [PubMed: 13376438]
24. Hagen V; Dekowski B; Nache V; Schmidt R; Gei üler D; Lorenz D; Eichhorst J; Keller S; Kaneko H; Benndorf K; Wiesner B, Coumarinylmethyl Esters for Ultrafast Release of High Concentrations of Cyclic Nucleotides upon One- and Two-Photon Photolysis. *Angewandte Chemie International Edition* 2005, 44 (48), 7887–7891. [PubMed: 16270373]
25. Hagen V; Bendig J. r.; Frings S; Eckardt T; Helm S; Reuter D; Kaupp UB, Highly Efficient and Ultrafast Phototriggers for cAMP and cGMP by Using Long-Wavelength UV/Vis-Activation. *Angew. Chem. Int. Ed. Engl* 2001, 40 (6), 1045–1048. [PubMed: 11268067]
26. Fournier L; Aujard I; Le, ÄÖSaux T; Maurin S; Beaupierre S; Baudin J-B; Jullien L, Coumarinylmethyl Caging Groups with Redshifted Absorption. *Chem.: Eur. J* 2013, 19 (51), 17494–17507. [PubMed: 24218195]
27. Gandioso A; Contreras S; Melnyk I; Oliva J; Nonell S; Velasco D; Garcia-Amoros J; Marchan V, Development of Green/Red-Absorbing Chromophores Based on a Coumarin Scaffold That Are Useful as Caging Groups. *J. Org. Chem* 2017, 82 (10), 5398–5408. [PubMed: 28467700]

Figure 1. Overview of PhotoActivated Depot (PAD) material designs.

Left column depicts first generation materials utilizing polymers to confer insolubility, and UV activated DMNPE group for photo release. Right column depicts the materials described in this work, utilizing a low molecular weight crosslinker to confer insolubility and using a visible light activated DEACM coumarin for photo release.

Figure 2. Synthesis of key intermediate CIMA.

Species 7, referred to as CIMA or coumarin insulin mono-azide, is ultimately formed through a carbamate linkage made between a coumarin alcohol and amine groups on the insulin.

Figure 3. HPLC and MS characterization of CIMA.

a) HPLC trace of the crude reaction product of insulin and intermediate 6 shows two prominent peaks. b) Purified CIMA isomer 1 HPLC trace. c) Purified CIMA isomer 2 HPLC trace. d) ESI-MS of isomer 1 (left) and isomer 2 (right).

Figure 4. Overview of MS analysis of isomers.

Overall sequence and connectivity of A and B chain of insulin shown, with cleavage sites for trypsin and GluC, as well as the three primary amine sites on the structure.

Figure 5. MS analysis of CIMA 2 isomer fragments after trypsin digestion.

Top) HPLC trace of CIMA-2 after trypsin digestion, showing three main fragments that were isolated and analyzed by ESI-MS. a) Fragment a ESI-MS spectrum, with a mass consistent with the unmodified fragment shown. b) Fragment b ESI-MS spectrum, showing a single coumarin addition. c) Fragment c ESI-MS spectrum showing coumarin addition on penultimate lysine.

Figure 6. MS analysis of CIMA 1 isomer fragments after GluC digestion.

Top) HPLC trace of CIMA-1 after GluC digestion, showing three main fragments that were isolated and analyzed by ESI-MS. a) Fragment a ESI-MS spectrum, with a mass consistent with the unmodified fragment shown. b) Fragment b ESI-MS spectrum, showing no modification. c) Fragment c ESI-MS spectrum showing coumarin addition on terminal amine of insulin A-chain.

Figure 7. Photolysis of CIMA isomers to release insulin.

CIMA isomer 1 and 2 independently photolyzed using a 406nm LED light source. Native insulin released was quantified by HPLC and plotted versus irradiation time. First order fits are shown, with the fit kinetic constant and standard error for the fit in parenthesis.

Figure 8. Synthesis of final insulin trimer PAD material.

4 equivalents of CIMA were mixed with the tri-dentate DBCO linker and reacted overnight to form the trimer. For clarity, only one site of attachment is shown explicitly. The remaining two are indicated with arrows.

Figure 9. Characterization of insulin trimer PAD material.

Top: HPLC trace of purified trimer PAD material, analyzed using size exclusion chromatography. Bottom: ESI-MS analysis of purified trimer.

Figure 10. Solubility of insulin and the insulin trimer PAD material in PBS. Solubilities were determined in triplicate and standard errors are shown.

Figure 11. Analysis of insulin trimer photolysis in DMSO/water.

Top: PAGE gel showing trimer before and during photolysis with a 406nm light source for the indicated period of time. Bottom: Final time point analyzed by ESI-MS, showing a mass consistent with native insulin.

Figure 12. Analysis of insulin released during continuous irradiation of insulin trimer in PBS by a 406nm light source.

Suspended particles of insulin trimer were irradiated by a 406nm light source. Insulin released into the supernatant was analyzed by HPLC and plotted versus irradiation time. The fit to a first order rate law is indicated, with the fit constant and its standard error (in parenthesis).

Figure 13. Analysis of insulin released during discrete irradiation of insulin trimer in PBS. Suspended insulin trimer was irradiated for 30 seconds at $t=0$ and $t=65$. Insulin released into the supernatant was analyzed and quantitated by HPLC and plotted versus time.

Figure 14. Thermal analysis of light source.

a) 406nm light source in place on animal, using a template that allows sequential irradiation of discrete locations. b) Sample thermal image of irradiated skin, immediately after light source has been removed. c) Quantified skin temperatures as a function of irradiation time, for both the low and high power light source. d) Skin 48 hours after irradiation for the indicated amounts of time (top is low power source, bottom is high power source). e) Histology of skin taken without irradiation (left) and 48 hours after 8 minutes of low power irradiation (right).

Figure 15. In-vivo analysis of insulin trimer.

30 second irradiation of skin using 406nm light source at t=0 and t=65 minutes. Control animals also irradiated, but light blocked with aluminum foil. (top) Blood insulin levels (M) as a function of time. (bottom) Blood glucose levels (%) as a function of time.