FULL TITLE: Biochemical Stabilization of Glucagon at Alkaline pH

RUNNING TITLE: Glucagon Formulation for Parenteral Use

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ABSTRACT

Background

For patients with type 1 diabetes mellitus, a bihormonal artificial endocrine pancreas system utilizing glucagon and insulin has been found to stabilize glycemic control. However, commercially available formulations of glucagon cannot currently be used in such systems because of physical instability characterized by aggregation and chemical degradation. Storing glucagon at pH 10 blocks protein aggregation, but results in chemical degradation. Reductions in pH minimize chemical degradation, but even small reductions increase protein aggregation. We hypothesized that common pharmaceutical excipients accompanied by a new excipient would inhibit glucagon aggregation at an alkaline pH.

Methods/Results

As measured by tryptophan intrinsic fluorescence shift and optical density at 630 nm, protein aggregation was indeed minimized when glucagon was formulated with curcumin and albumin. This formulation also reduced chemical degradation, measured by liquid chromatography with mass spectrometry. Biological activity was retained after aging for 7 days in an *in vitro* cell-based bioassay and also in Yorkshire swine.

Conclusions

Based on these findings, a formulation of glucagon stabilized with curcumin, polysorbate-80, L-methionine, and albumin at alkaline pH in glycine buffer may be suitable for extended use in a portable pump in the setting of a bihormonal artificial endocrine pancreas.

INTRODUCTION

Glucagon raises blood glucose through activation of glycogenolysis and gluconeogenesis (1), but its secretion in response to hypoglycemia is abnormal in type 1 diabetes (2). The inclusion of glucagon within a dual-hormone, closed-loop control system for treatment of type 1 diabetes limits hypoglycemic events (3-7). However, commercially-available glucagon, a lyophilized solid, rapidly aggregates after reconstitution with water, forming high-molecular-weight complexes (8-10), making it unsuitable for extended use in a closed-loop system. Aggregated glucagon is cytotoxic at high concentrations (11, 12) and its action *in vivo* is delayed compared to fresh glucagon (12). Methods that show promise for stabilizing glucagon include formulation in alkaline media (12), creation of mutant glucagon analogs (13), the use of surfactants to increase solubility at neutral pH (14), and the use of anhydrous solvents to minimize chemical interactions with water (15).

Our previous work examined chemical degradation of glucagon at alkaline pH, which include deamidation and oxidation (16). At pH 9, glucagon aggregates more but degrades much less than at pH 10; therefore, the use of pH 9 would require the addition of anti-aggregation excipients. To address this issue, we report here on the effects of commonly used excipients and the novel use of curcumin. Curcumin is a polyphenol derived from *Curcuma longa* rhizomes that exhibits multiple biological actions (17-22) and has shown promise in reducing aggregation of neurodegenerative peptides important in Alzheimer's disease and Parkinson's disease (23-30).

In this report, we undertook a series of experiments that examined the pH dependence of glucagon aggregation, the effects of single and multiple excipients to block glucagon aggregation, the biological activity of formulations in a cell-based bioassay, and the

pharmacokinetic (PK) and pharmacodynamic (PD) behavior of fresh and aged stabilized glucagon in swine.

MATERIALS & METHODS

Reagents

L-arginine, anhydrous sodium sulfite, anhydrous D-(+)-lactose, anhydrous glycerol, polysorbate-80, hydrochloric acid, anhydrous D-trehalose, trimethylamine N-oxide dihydrate, curcumin, Thioflavin T, HyClone-modified Dulbecco's phosphate-buffered saline (DPBS), curcumin, bovine serum albumin (BSA), and sodium hydroxide were purchased from Thermo Fisher Scientific, Inc. (San Jose, CA). Human serum albumin (HSA), 2-hydroxypropyl-β-cyclodextrin, L-methionine, and dimethyl sulfoxide were purchased from Sigma-Aldrich (Saint Louis, MO). Polyvinylpyrrolidone was kindly donated by BASF (Kollidon 17 PF; Ludwigshafen, DE) and ISP Technologies (Plasdone C17; Wayne, NJ). Native human glucagon was synthesized by AmbioPharm, Inc. (Beech Island, SC) as reported previously [16]. The peptide was purified to >99% and shipped as a lyophilized solid. Reagents were of highest purity available.

Sample formulation and aging

Stock glycine buffer was pH-adjusted with sodium hydroxide. The final concentration of both glucagon and HSA was 1 mg/ml. Prior to its use, solutions were passed through a 0.2-μm PVDF filter. Curcumin-containing solutions were formulated with gentle heating to bring curcumin into solution without the use of a solvent [31]. A stock glycine buffer was diluted to ~80% of the final volume, brought to a pH of ~12, then heated to 95-100°C. Curcumin was added to the solution and allowed to stir at 4°C until the solution reached room temperature (~30

min). The pH was then brought to 9 using dilute hydrochloric acid and excipient solutions were added. The solution was then brought to the final volume, glucagon and/or HSA added, and passed through a 0.2-µm PVDF filter into a glass vial, which was then sealed. For optical studies (TIF, OD630, and TEM), a very low concentration of DMSO (0.5% v/v) was used to assist in solubilizing the curcumin.

All formulations were placed into a static 37°C incubator for the aging period. Unaged samples were immediately placed into a -20°C freezer. After aging was completed, the samples were frozen at -20°C until analysis.

Assays for peptide aggregation

The following assays were used to assess the effect of pH on glucagon aggregation and the ability of excipients to reduce aggregation.

Thioflavin T (ThT) fluorescence assay

ThT fluorescence quantifies protein aggregation (32). A ThT stock solution was prepared by adding 8 mg of solid ThT to 10 ml of HyClone DPBS and shaking overnight in the absence of light. To prepare the working solution, the stock solution was passed through a 0.2 µm pore-size filter to remove insoluble particulates from the ThT solution then diluted 1:50 with HyClone DPBS and used immediately. For analysis, 10 µl of sample or blank were pipetted into a black 96-well plate (Greiner Bio-One; Monroe, NC) followed by the addition of 200 µl of the ThT working solution to each well. A SPECTRAmax GEMINI microplate reader (Molecular Devices Corporation, Sunnyvale; CA) was used with excitation/emission wavelengths of 442/482 nm.

Tryptophan intrinsic fluorescence (TIF) assay

It has previously been shown upon aggregation of glucagon that tryptophan 25 experiences a blue-shift that can be used to assess overall peptide aggregation (8). This

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tryptophan 25 is located in a C-terminal hydrophobic patch that has been implicated in glucagon

aggregation so we feel this assay is suitable because tryptophan 25 is most likely involved in

glucagon aggregation and undergoes this blue-shift. As the peptide aggregates, there is

increasing signal at 320 nm and decreasing signal at 350 nm. For analysis, excitation was at 280

nm and the end point (TIF shift) was emission at 320 nm minus emission at 350 nm.

It has been reported that ThT assays cannot be used for curcumin-containing solutions

due to optical interference from curcumin (33) and other small fluorescent organic molecules

(34) due to curcumin's absorbance at 425 nm, a region similar to the emission wavelengths of

aggregate-bound ThT. To verify this effect, we obtained samples of highly aggregated glucagon

and carried out ThT assays with this material alone and immediately after mixing with 1 mM

curcumin. When curcumin was added to this sample, the ThT data immediately showed a large

(66%) loss of signal, indicating quenching by curcumin. The same experiment using the TIF

assay showed no loss of signal (the TIF spectrum is much lower than curcumin absorbance).

Thus, for curcumin experiments, we report TIF shift but not ThT data.

Optical density at 630 nm

When glucagon aggregates, it forms insoluble gels that impart cloudiness to solutions and

can be measured by light scattering or light obscuration (14). By measuring the optical density at

630 nm, absorbance due to curcumin is minimized. Into a clear 96-well plate (Greiner Bio-One),

200-μl samples of sample or blank were aliquoted, shaken gently, and read at 630 nm in an

Epoch UV-visible spectrophotometer plate reader (BioTek; Winooski, VT). Data were corrected

for path length differences among wells.

Effect of surfactant on protein stability and solubility

To assess the effect of polysorbate-80 on glucagon solubility, a Bradford protein assay (Pierce; Rockford, IL) was performed using 0%, 0.1%, 0.5%, 1%, 2.5%, and 5% v/v of polysorbate-80 concentrations. The concentration of polysorbate-80 required was defined as the lowest concentration necessary to reach a total protein concentration in the formulation of 2 mg/ml.

Fluorescence bioassay: protein kinase A

The glucagon bioassay used for these experiments employs CHO-K1 cells that stably overexpress the human glucagon receptor and also express an enhanced green fluorescent protein-protein kinase A fusion protein (GFP-PKA) reporter molecule (35), as previously reported (16, 36). Briefly, after overnight culture, the cells were exposed to serially diluted glucagon in culture medium without antibiotics for 30 min at 37°C/5% CO_2. Cells were then fixed in 10% formalin for 20 min, washed, labeled with 1 μM Hoechst DAPI in PBS for 30 min, imaged with a Zeiss Axiovert 200M fluorescence microscope (Intelligent Imaging Innovations, Inc.; Denver, CO), and data analyzed with Slidebook 5.0 (Intelligent Imaging Innovations). Loss of fluorescence is the measure of the cellular response to glucagon. The EC_{50}, a metric of potency, in pg/ml, was determined as the midpoint between the 100% plateau and the response floor using a 4-parameter logistic model $\left(y = A + \left[\frac{B-A}{1+10^{\frac{C-x}{D}}} \right] \right)$ in the XLfit software program.

Liquid chromatography-mass spectrometry (LCMS)

Glucagon formulated with different excipients was analyzed by LCMS. Samples were diluted to 1 μM in 1% formic acid from 1 mg/ml and held at 4°C until analysis. Ten picomoles of protein were injected into an Agilent 1100 high-performance liquid chromatography (HPLC) system fitted with a reverse-phase ZORBAX SB-C18 column (Agilent Technologies; Santa

Clara, CA). A mobile phase of 1% formic acid and a gradient of acetonitrile from 7.5-45% over 60 minutes were used. Mass spectrometry analysis was performed in a Thermo linear ion trap Velos instrument (Thermo Fisher Scientific). Peptide MS analysis and identification was performed using Xcalibur 2.2 software (ThermoFisher Scientific).

Unmodified glucagon has a monoisotopic molecular weight of 3,480.6 Da. Detection of oxidized or deamidated glucagon was carried out by examining species that had a molecular weight increase of +16 or +1 Da, respectively. Other modifications were determined from the mass spectrum using Xcalibur or other proteomics tools. For all LCMS figures, retention time shift refers to setting unmodified glucagon retention time as 0 minutes (experimentally ~29.5 minutes). It should be noted that polysorbate-80 will saturate the detection signal when analyzed via LCMS. For this reason, we chose to analyze the final formulations without polysorbate 80 to learn about the chemical and physical degradation in our formulation.

Transmission electron microscopy (TEM)

A 10-µl sample including 1 mg/ml glucagon with or without 1 mM curcumin and 1 mg/ml HSA (pH 9) was deposited on a copper grid, stained with 1.33% uranyl acetate for 45 seconds, air-dried, and viewed at100 kV using a Philips CM120 microscope. Images were collected as 1,024x 1,024 pixel files on a Gatan 794 CCD multiscan camera (Gatan, Inc.; Pleasanton, CA) and converted into TIFF images (Image-J, NIH). Sixty images for each condition were each examined by three blinded observers.

Yorkshire swine study: pharmacokinetic and pharmacodynamic evaluation

Each of 11 Yorkshire pigs was tested with two formulations of glucagon over several months for a total of 22 experiments. The Oregon Health and Science University Institutional Animal Care and Use Committee approved the protocol (ID: IS00001790). The formulations

tested were unaged curcumin-stabilized glucagon and curcumin-stabilized glucagon aged for 7 days at 37°C. Overnight fasted pigs were sedated using Telazol and atropine, weighed, intubated, and maintained on 1.5-2.0% isoflurane and oxygen. In order to block endogenous pancreatic α and β-cell function, each animal was given octreotide (3 µg/kg/hr IV) starting 40 min prior to the glucagon bolus. The glucagon dose was given subcutaneously at time zero in a dose of 2µg/kg. Venous blood glucose was monitored in real time every 10 min for 2 hours (HemoCue 201 analyzer, Cypress, CA). Venous blood samples were also drawn periodically for glucagon and insulin concentration measurement. Glucagon was analyzed by RIA (EMD Millipore; St Charles, MO) and insulin by ELISA (Mercodia AB; Uppsala, Sweden).

For pharmacokinetic and pharmacodynamic data, 4 parameters were determined: the maximum change in glucagon concentration or glucagon effect, time to early half maximum concentration or effect, time to maximum change of concentration or effect, and 60-min incremental area under the curve (AUC) of concentration or effect. Pharmacodynamic data fit a normal distribution and thus parametric statistics were applied to PD data analysis. Pharmacokinetic (PK) data was significantly skewed (24 of 28 tests) for each metric; for this reason, we analyzed PK data by non-parametric median-based statistics.

Statistical Tools

Pharmacodynamic data was analyzed by a two-sided Student's t-test while pharmacokinetic data was analyzed by a Mann-Whitney U-test, both with significance defined as $\alpha < 0.05$. Results are presented as mean ± SEM (PD) or median with 25th and 75th percentiles (PK).

RESULTS

Glucagon aggregation increases substantially below pH 9.7

The effect of pH (8.5 to 9.8) on aggregation kinetics over 72 hours is shown in Figure 1A for ThT and in Figure 1B for TIF shift. The more alkaline samples (pH 9.7 and 9.8) showed very little aggregation by ThT or TIF over this time period. However, substantial changes in ThT and TIF shift were found within a narrow pH range; there was little evidence of aggregation at pH 9.7, but substantial aggregation at pH 9.5. After reaching a maximum, the fluorescence tended to decline or plateau, especially for ThT.

Curcumin, HSA, and tween 80 inhibit glucagon aggregation

In the TIF assay, the presence of tween 80, HSA, and curcumin in glucagon aged for 7 days at 37°C greatly reduced aggregation (Figure 2A and B). One mM curcumin substantially reduced aggregation, but 0.01 mM curcumin was not effective. When HSA was added to preparations with curcumin, aggregation at 3 and 7 days was significantly reduced compared to no excipient.

Figure 2C shows optical density (OD_{630}) measurements on the same samples in Figure 2B. These data also demonstrate that the curcumin and HSA block age-related aggregation.

Transmission electron microscopy verifies the anti-fibrillation effect of curcumin

Glucagon samples were aged for 14 days rather than 7 days to allow more time for visible aggregates to occur. Upon examination of the aggregates formed in alkaline glucagon, it was seen that under these conditions, glucagon forms distinct yet short fibrils, as seen in Figure 3A. All observers identified fibrils in samples without excipient and none found fibrils in samples containing curcumin and HSA. For glucagon without excipient, the 3 observers found fibrils in 7

of 60, 8 of 60 and 8 of 60 (p < 0.001, Fisher's exact test). The Fleiss kappa result for the observers was 0.86 (z = 20), indicating very high concordance among the 3 observers.

Figure 3A shows glucagon fibrils without excipient after 14 days. Figure 3B shows absence of fibrils in a specimen in which glucagon was aged with curcumin and HSA. Each of these images is representative of the entire set of analyzed micrographs.

Glucagon oxidation from curcumin

LCMS measured glucagon chemical degradation during aging. Minimal chemical degradation occurred when glucagon was aged for 7 days at pH 9 at 37°C, as seen in Figure 4B, although aggregation was marked under this condition. When curcumin was added at 1 mM to block aggregation, almost complete oxidation of glucagon occurred along with modest deamidation (Figure 4C). To avoid oxidation of glucagon, L-methionine (1 mg/ml) was added. The L-methionine markedly reduced oxidation, although adducts of glycine and glucagon appeared within the chromatogram, seen at +1 to 2 min (Figure 4D). HPLC showed minimal chemical or physical degradation due to polysorbate-80 (data not shown). Oxidation at the level found after L-methionine addition does not seem to affect aggregation of glucagon, there still is significant aggregation occurring as shown by TIF (supplemental figure 3).

Other excipients to stabilize curcumin and polysorbate-80 to increase glucagon solubility

Polysorbate-80 was successful in increasing solubility of glucagon and curcumin. Without polysorbate-80, the protein (glucagon and HSA) was found to precipitate at 4°C and -20°C. The addition of polysorbate-80 yielded a clear solution without precipitate. This finding was confirmed by UV-visible spectrophotometric analysis for curcumin and the Bradford assay for protein content, both of which showed maintenance of initial concentration (Supplemental Figure 2). Polysorbate-80 has the added benefit of helping to reduce aggregation as well, as

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shown in Figure 2A. HSA was added because of reports that common mammalian serum proteins, particularly albumin, stabilize curcumin (37, 38).

Curcumin reduces age-induced potency loss in glucagon

Glucagon formulated at pH 9 and aged for 7 days at $37^{\circ}C$ showed a significant loss of potency (increased EC_{50}) vs unaged glucagon ($p < 0.001$, Figure 5A) in a cellular bioassay for glucagon receptor activation. In contrast, when the curcumin-stabilized glucagon formulation (including 1 mg/ml HSA, 1 mg/ml L-methionine, and 0.5% v/v polysorbate-80) was tested, the EC_{50} shift between the aged and unaged preparations was much smaller and not significantly different from one another ($p = 0.33$). In addition, the increase in EC_{50} over time was significantly greater in the glucagon formulation without excipient than in the curcumin-stabilized glucagon formulation ($p = 0.008$). It should also be noted that reagent controls without glucagon do not elicit any biological response (i.e. EC_{50} is infinite).

Evaluation of curcumin-stabilized glucagon in swine

Yorkshire pigs were given subcutaneous injections of unaged curcumin-stabilized glucagon and aged curcumin-stabilized glucagon (same formulation as in cell bioassay) to measure PK/PD. Insulin levels stayed within 10-20% of baseline over the 3-hour period of each study, demonstrating that the insulin response to hyperglycemia was appropriately inhibited by octreotide (data not shown). Figure 6 shows the change in blood glucose rise over time for the two preparations. In terms of the hyperglycemic responses, there were no significant differences between fresh and aged curcumin-stabilized preparations at any time point.

Additional metrics, including early $t_{50\% \, max}$ and t_{max} were also used to compare the glucose and plasma glucagon concentrations (Table 1). Since each of the 11 animals was given both formulations, paired tests were used; no differences between unaged vs aged formulation

were seen. Additionally, no edema or erythema was seen in any swine over the period of the examination (180 min).

Effect of a variety of excipients on glucagon degradation and bioactivity

Many other excipients were tested for their potential to stabilize glucagon (Supplemental Table 2). Several excipients led to substantial deamidation and/or oxidation. Some caused chain cleavage or adduct formation (data not shown). The potency loss for several (L-arginine, β-cyclodextrin, and trimethyl amine N-oxide) was marked. In general, these excipients were ineffective in blocking aggregation (data not shown) and were excluded from further testing.

DISCUSSION

The effect of curcumin on glucagon aggregation

We chose to test curcumin as an excipient for glucagon formulation because of its ability to reduce amyloid fibrillation in Alzheimer's disease and Parkinson's disease (23-30). We added HSA to the solutions because of published evidence that it reduces spontaneous degradation of curcumin (37). We found that high concentrations of curcumin yielded a solution that was free of assayable aggregates, even without the addition of HSA. The persistent benefit of the high concentrations of curcumin is likely due to weak bonding interactions that disrupt the secondary structure characteristic of protein aggregates. Curcumin contains two aromatic moieties that would participate in π-π electron stacking with aromatic amino acids (27). The aromatic residues of glucagon are located near hydrophobic patches at the N and C termini (residues 6-10 and 23-27), which likely contribute to its tendency toward aggregation (39). The phenyl moieties of curcumin could disrupt self-association (aggregation) of these hydrophobic patches. In addition,

Reinke *et al.* postulated that the conformational shape and the length of the hydrocarbon linker within curcumin assist in disrupting amyloid formation (40).

Curcumin leads to glucagon oxidation, which is inhibited by L-methionine

The pro-oxidant activity of curcumin (19) probably accounts for the glucagon oxidation found by LCMS. As we found, Met27 is oxidized during the aging of glucagon (16). L-methionine, which likely undergoes sacrificial oxidation, markedly reduced glucagon oxidation. The glycine adducts that form with L-methionine are not well understood; it is possible that other phenolics may stabilize glucagon without formation of these adducts.

Stabilized glucagon is bioactive *in vitro* and *in vivo*

Given glucagon aggregation delays its absorption (12) and leads to cytotoxicity (11), aggregation must be addressed. In the cell-based bioassay, there was significant loss of potency in aged vs unaged glucagon without excipient. In contrast, aged curcumin-stabilized glucagon only exhibited a minimal, non-significant loss of potency. This finding strongly suggests that curcumin maximizes the glucagon monomeric (bioactive) conformation.

Aged and unaged curcumin-stabilized glucagon was also tested in octreotide-treated pigs; no significant difference between unaged and aged stabilized glucagon in PK or PD parameters was found, which suggests minimal loss of bioactive glucagon. The 2 µg/kg dose we gave to pigs is a submaximal dose, much different than the massive dose in the human hypoglycemia kit. For this reason, we believe that if there was degradation of glucagon in the 2 µg/kg dose, there would be loss of the biological effect (we observed no PK or PD loss). In fact, one reason that we chose this submaximal dose of the glucagon for the pig study was so that we could detect a loss of effect if there was degradation.

Current commercially-available preparations of glucagon are acidic, lyophilized formulations that must be used immediately after reconstitution in order to minimize exposure to aggregated glucagon. Our studies here suggest that formulating glucagon at a pH of 9 with the addition of curcumin and albumin may well extend the usage period to 7 days, which would be useful in a bihormonal artificial endocrine pancreas system.

Our previous work described several chemical degradations of glucagon at alkaline pH during aging (16). Acidic glucagon formulations form a variety of fibrillar polymorphisms (10), but glucagon formulated at pH 10 remains monomeric (12). Although glucagon, like other hormones, is stored in amyloid fibrils in its membrane-bound secretory granules (41), aggregation of the drug must be minimized for exogenous administration via a pump.

pH dependence of glucagon aggregation

Though glucagon is highly degraded at pH 10, less physical and chemical degradation occurs at pH 9 (16). Here we extend those findings and show that there is a narrow zone between pH 9 and 10 at which and below which glucagon aggregates. As pH is lowered, aggregation occurs more rapidly; at pH 8.5, glucagon begins to aggregate within 12-14 hours. Though the alkaline range might seem unusual for drug formulation, there are many pharmaceuticals formulated in this range; such as amitriptyline and its metabolite nortriptyline, trimipramine, mianserin, mirtazapine, citalopram, paroxetine, sertraline, venlafaxine, levomepromazine, quetiapine, ketobemidone, tramadol, alimemazine, and metoprolol (42) Most are oral administration but amitriptyline, levomepromazine, ketobemidone, tramadol, and metoprolol can be formulated as injectable solutions, with levomepromazine specifically listed as subcutaneous. In addition, we found that a solution of albumin when prepared at a pH of 10 did not induce more discomfort in humans compared to a pH of 7.4 (43); speed of injection was a contributing

factor. Though not tested in this study, this earlier finding suggests that the current formulation of glucagon (pH 9) may be pain-free upon injection.

Implications, limitations, and future studies regarding curcumin-stabilized glucagon

Though additional regulatory work is needed for verification, it appears that stabilization of glucagon by curcumin might well be a promising step for treatment of persons with type 1 diabetes, since the formulation was active *in vitro* and *in vivo*.

Limitations of this study include lack of shelf-life stability experiments to examine long-term stability. Another limitation is that curcumin is relatively uncharacterized when it comes to the pharmaceutical industry. Product searches revealed that its primary use currently is in unregulated nutraceuticals. The regulatory approval process for curcumin might well be cumbersome. Related compounds (congeners) (18) may facilitate a faster regulatory process and may have increased anti-aggregation attributes compared to curcumin. Additionally, in terms of our *in vivo* studies, we only studied one dose; multiple doses will be needed to measure power and potency.

In conclusion, we have described the development of a stabilized formulation of the amyloid forming peptide, glucagon, through the use of curcumin, albumin, an antioxidant and a surfactant, buffered to pH 9. This preparation shows minimal protein aggregation, minimal chemical degradation, and high activity both in a cell-based bioassay and in live pigs.

AUTHOR CONTRIBUTIONS

Authors Caputo and Ward are the guarantors of this work and, as such, had full access to all the data in the study. They take full responsibility for the integrity of the data and the accuracy of the data analysis. WKW, JRC, MAJ, NC, JEY, and CTR conceived of, and planned the project. NC, CPB, JEY, PAB, MAJ, LLD, GLL, MEB, and JMC researched and/or collected data; NC, MAJ, CPB, JRC, JEY, LLD, CTR, JMC, and WKW performed data analysis; NC, MAJ, JRC, CTR, and WKW wrote the manuscript.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflicts of interest for the work described.

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FIGURE LEGENDS

Figure 1: Fluorescence spectroscopy of glucagon aggregation in the pH range 8.5 to 9.8 over a period of 72 hours at room temperature. **A)** Aggregation was assayed by the use of the Thioflavin T fluorescent probe that quantitatively binds to aggregated protein. Between pH 9.5 and 9.7, aggregation was greatly reduced, with the peak fluorescence at pH 9.7 and 9.8 showing no significant difference in fluorescence from time = 0 hours. **B)** Aggregation was assayed by measuring the tryptophan fluorescence at 320 minus fluorescence at 350 nm. Results similar to Figure 1A were found; there was a definite shift in aggregation between pH 9.5 and 9.7 with pH 9.7 and 9.8 peak fluorescence shift showing no significant difference from time = 0 hours. N refers to replicates per plate tested.

Figure 2: Aggregation assays showing the effect of excipients to inhibit aggregation. **A)** Tryptophan intrinsic fluorescence shift showing both HSA and tween 80 reduce aggregation over a period of 7 days while L-methionine does not help aggregation significantly. B) High levels of curcumin at pH 9 minimize glucagon aggregation and are significantly lower in aggregation after 7 days at $37^{o}C$ compared to glucagon without excipient at pH 9. The benefit of HSA was also seen since 0.1 mM curcumin by itself shows no major benefit but when aged along with HSA there was significantly lower aggregation at 3 and 7 days of aging at $37^{o}C$. **C)** Solution optical density measured at 630 nm, which related to physical gelation of glucagon. The trends seen in panel C were similar to those seen in panel B. N refers to replicates per plate tested.

Figure 3: Transmission electron micrographs at 4,800x magnification, showing **A)** 1 mg/ml glucagon without curcumin aged for 14 days showing densely-packed fibrils and **B)** 1 mg/ml glucagon with curcumin aged for 14 days shows an absence of fibrils.

Figure 4: High performance liquid chromatograms with mass spectrometry (LCMS) of different glucagon preparations. **A)** Unaged glucagon at pH 9 shows a well-defined peak that is identified as native glucagon. Minor amounts of chemical degradation may have occurred as a result of long term storage. **B)** Native glucagon aged for 7 days at 37°C at pH 9 shows minimal chemical degradation (though this preparation was markedly aggregated as shown in Figure 1 and Figure 2). **C)** Addition of curcumin causes near complete oxidation of glucagon. **D)** Addition of L-methionine to the glucagon/curcumin solution prevents the curcumin-induced oxidation, although there are additional products that are formed at ~+1.5 min that can be attributed to glycine adducts (mass equal to glycine added to glucagon).

Figure 5: Glucagon bioassay based on PKA activation. Dose-response curves showing EC$_{50}$ shifts. **A)** 1 mg/ml glucagon prepared at pH 9 and aged for 7 days at 37°C shows a significant change in potency. **B)** 1 mg/ml glucagon prepared with 1 mM curcumin, 1 mg/ml HSA, 1 mg/ml L-methionine, and 0.5% v/v polysorbate-80 shows no significant change in potency over 7 days aged at 37°C. The fold change in potency for the curcumin-stabilized glucagon shows significant difference from the pH 9 fold change in potency. N refers to the number of individual experiments performed.

Figure 6: Mean pharmacodynamic (PD) effects of different glucagon formulations given subcutaneously in Yorkshire pigs. Results from fresh curcumin-treated glucagon, and results from 7-day aged curcumin-treated glucagon show no significant difference at each time point. N refers to the number of individual swine tested.

Table 1: PK/PD metrics determined from this study. There were no significant differences among both preparations. Max Δ = average maximum change in venous glucose or plasma

glucagon; early $t_{50\%}$ = average time to 50% of maximum before peak is reached; t_{max} = average time to maximum change; 60 min AUC_{inc} = incremental area under the curve for metric. PD data analyzed according to parametric statistics; PK data was significantly skewed (24 of 28 tests) for each metric and analyzed by non-parametric statistics.
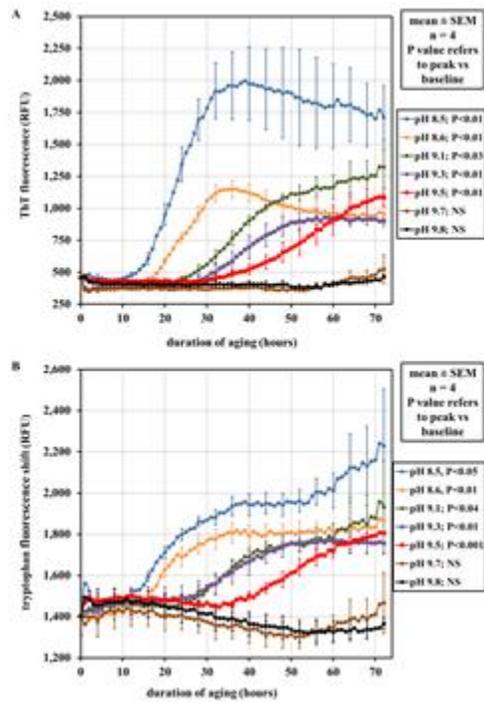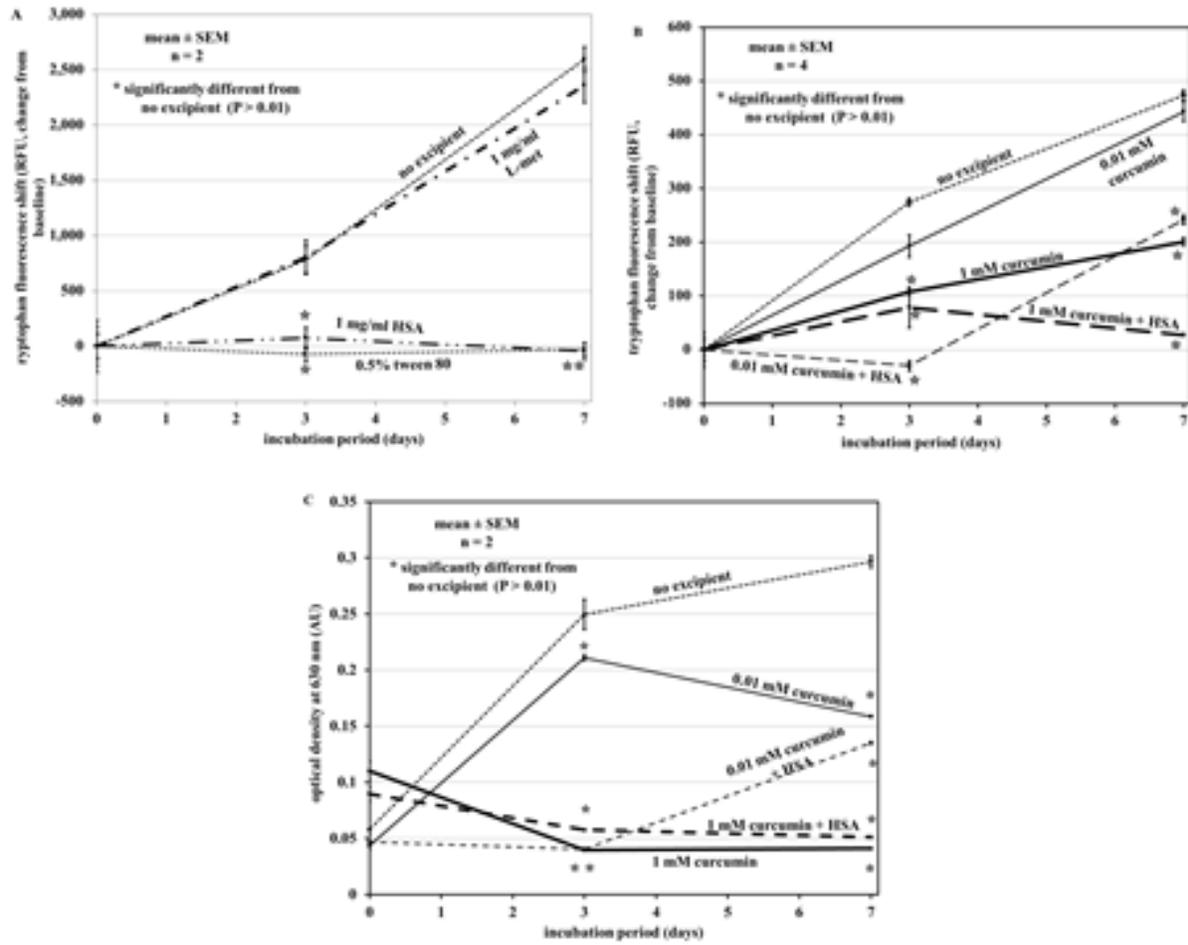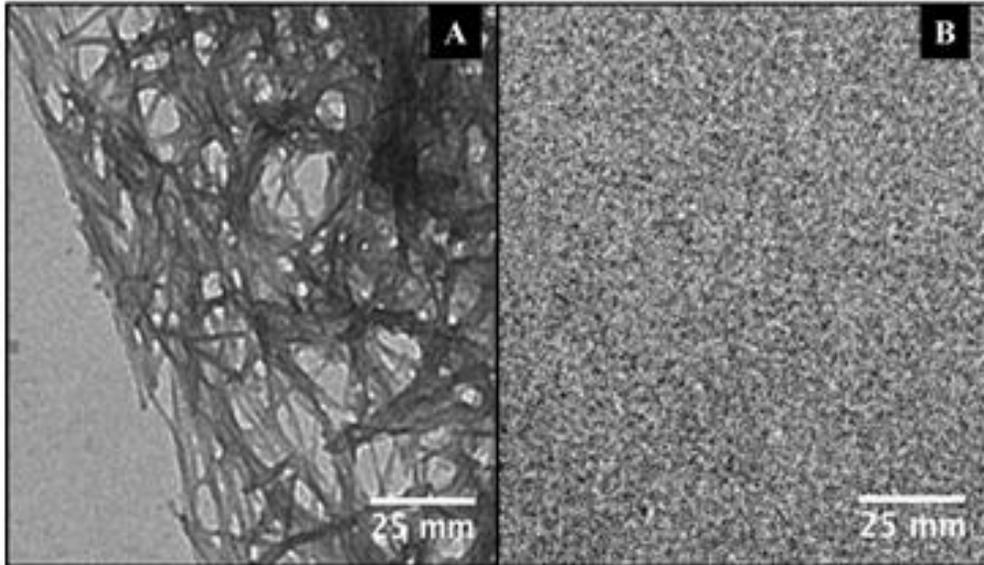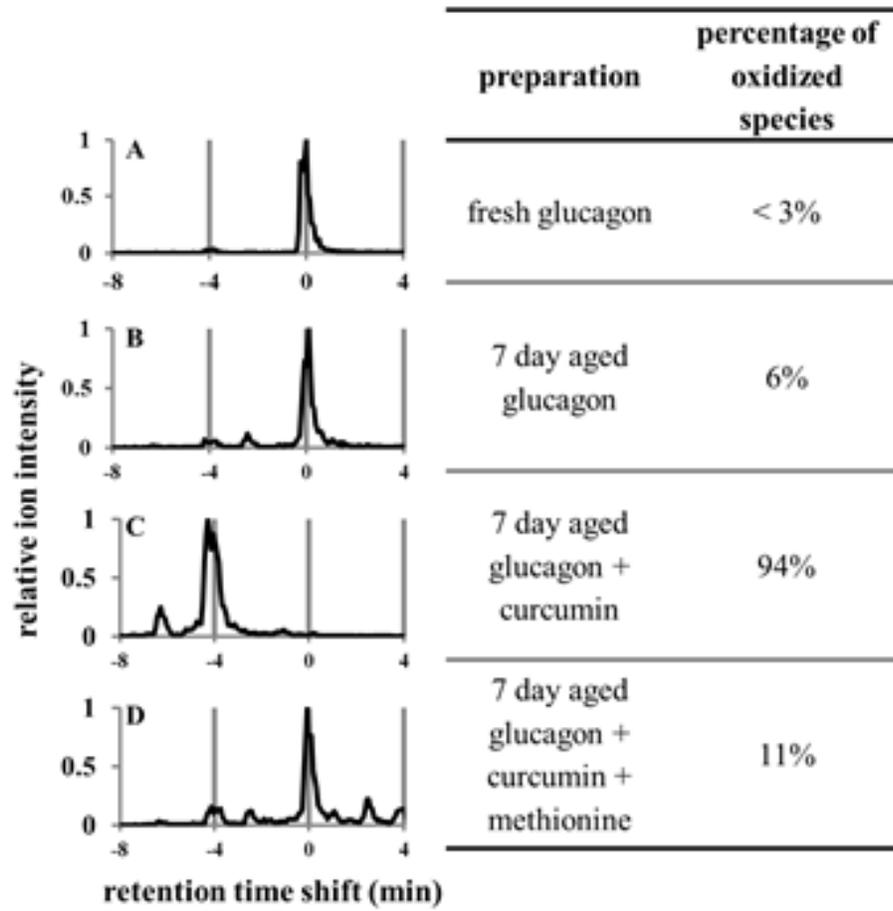
FIG 1:

FIG 2:

FIG. 3:

FIG 4:

	preparation	percentage of oxidized species
A	fresh glucagon	< 3%
B	7 day aged glucagon	6%
C	7 day aged glucagon + curcumin	94%
D	7 day aged glucagon + curcumin + methionine	11%

FIG 5:
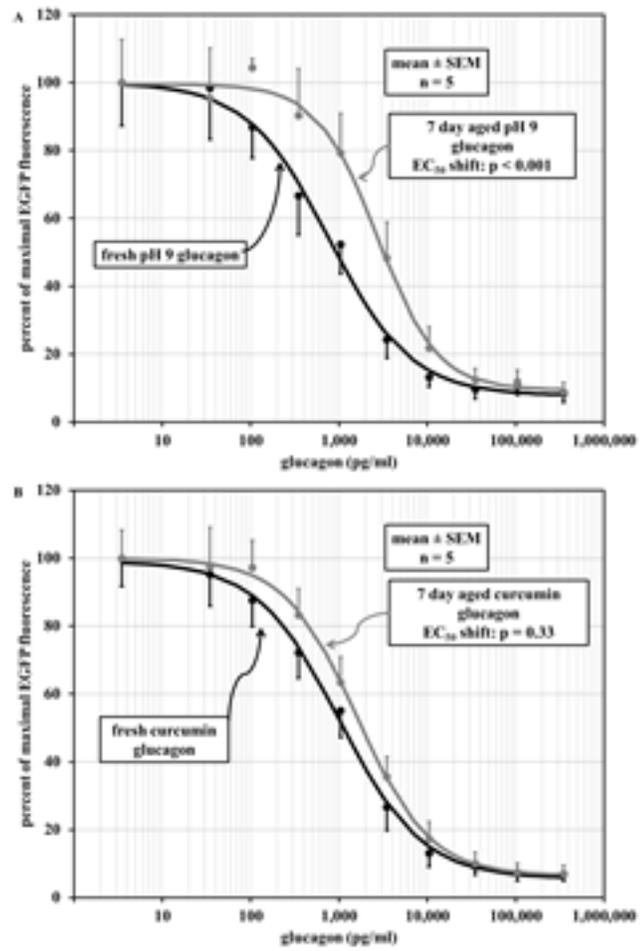
FIG 6:
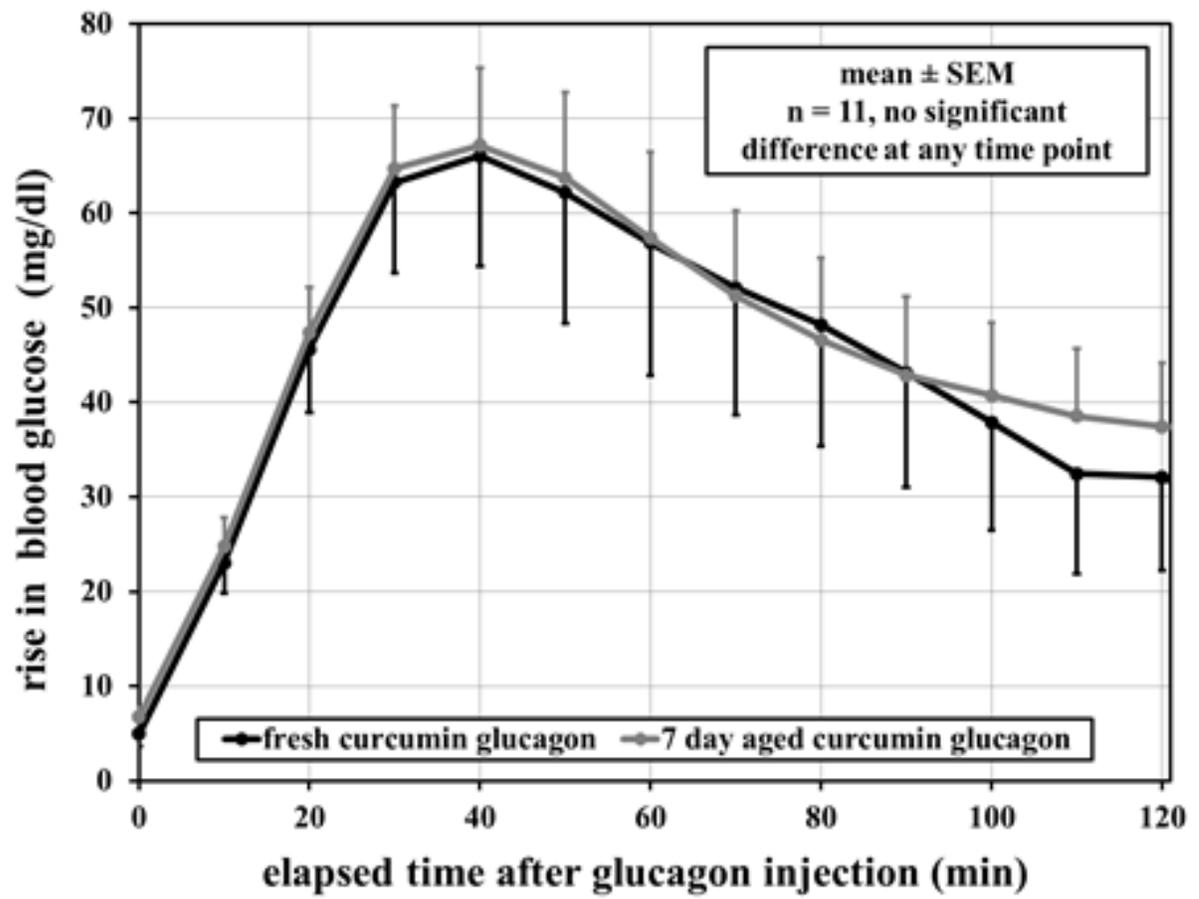
FIG 7:

	metric (n = 11)	fresh curcumin glucagon	7 day aged curcumin glucagon
venous glucose (PD; mean ± SEM)	max Δ (mg/dl)	70 ± 12	70 ± 8
	early $t_{50\%}$ (min)	15 ± 1	14 ± 1
	t_{max}, (min)	40 ± 4	36 ± 2
	60 min AUC_{inc} (min*mg/dl)	2,801 ± 501	3,002 ± 339
plasma glucagon (PK; median [25%:75%])	max Δ (pg/ml)	278 [220:586]	359 [259:2,596]
	early $t_{50\%}$ (min)	9 [4:12]	5 [3:7]
	t_{max}, (min)	30 [20:70]	20 [20:30]
	60 min AUC_{inc} (min*pg/ml)	12,611 [9,188:20,491]	14,993 [10,758:114,577]