Synthesis and Evaluation of the Insulin–Albumin Conjugate with Prolonged Glycemic Control

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ABSTRACT: Engineering therapeutic proteins to improve their half-life so as to sustain physiologically relevant extended activity is the need of the hour in biopharmaceutical research. In this study, insulin and bovine serum albumin (BSA) were independently functionalized rationally and were later conjugated to prolong the half-life of insulin. The thiol functionalization of BSA with 2-iminothiolane in the ratio 1:20 yielded an average of 6–8 thiols/BSA, which then reacted with maleimide-functionalized insulin to form an insulin–albumin conjugate. The bioconjugate was purified by size exclusion chromatography, and the increase in size was confirmed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis. Bioconjugation resulted in a multi-fold increase in the hydrodynamic volume of the insulin–albumin conjugate as measured in DLS when compared to BSA. The glucose uptake assay with 3LT3-L1 cell lines was performed, and the mean fluorescence intensity (MFI) of 16.16 observed for the insulin–albumin conjugate was comparable to insulin (19.42). The blood glucose reducing capacity of the insulin–albumin conjugate in streptozotocin induced diabetic male Wistar rats was well maintained up to 72 h when compared to native insulin. Further, a three-fold increase in plasma insulin concentration was observed in bioconjugate treated animals as against insulin treated animals after 24 h of treatment using ELISA. The histological analysis of different organs of the bioconjugate treated rats indicated that it was non-toxic. This study has paved a way for further detailed studies on similar bioconjugates to develop next-generation biotherapeutics for treating diabetes.

INTRODUCTION

Diabetes mellitus is a global epidemic caused by either defects in insulin production or insulin action or both, resulting in high blood glucose levels. According to the World Health Organization, diabetes is one of the four major non-communicable diseases, and its prevention is of paramount interest (World Health Organization 2016). At present, 463 million people are living with diabetes and the cases are expected to increase up to 700 million by 2045, according to the international diabetes federation (IDF 2019). The major classes of diabetes are type-1 and type-2 diabetes, which are more prevalent in the world. Type-1 diabetes is usually an autoimmune disease where the pancreatic beta cells are destroyed by the immune system. Due to this there is no production of insulin, and the onset of the disease is observed mostly in the young individuals. In contrast, type-2 diabetes is an effect of both environmental factors and genetics. Usually the onset of type-2 diabetes is late and is observed in adults. Treatments for type-1 and type-2 diabetics vary with the phase and progression of the disease. In the case of type-1 diabetes, insulin is the only medication prescribed to control diabetes, whereas in the case of type-2 diabetes, treatment starts with the use of biguanides (metformin), insulin secretagogues (sulfonylureas and meglitinides), insulin sensitizers (thiazolidinediones), and finally, insulin is used as a last resort. Since the discovery of insulin, it has been used for treating diabetes starting with the use of bovine insulin; with the advances in the field, many insulin analogues have been produced. The use of the recombinant DNA technology was a breakthrough in producing the modern insulin analogues like glargine, aspart, lispro, and so forth. The use of new insulin analogues in the last two decades has revolutionized the treatment regimens. Although insulin analogues have made considerable strides in the treatment of diabetes, there is still a need for a better insulin analogue with increased half-life.

Many strategies have been used to increase the half-life of proteins such as increasing their hydrodynamic volume by PEGylation, glycosylation, conjugation with albumin, FC-fusion proteins, and so forth to reduce renal clearance and proteolysis. Similarly, insulin has been modified in numerous ways since its discovery, few of them are PEGylation, derivatization with fatty acids for in vivo albumin binding.

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covalent conjugation with albumin,\textsuperscript{16,17} and polymer-based encapsulations.\textsuperscript{18} Albumin conjugation is of particular interest as albumin is the most abundant plasma protein with a half-life of 19 days and is known for its significant functions such as transport of biomolecules and metal ions, maintenance of colloidal osmotic pressure in blood, and drug binding affinities.\textsuperscript{19–21} It has been used to increase the pharmacokinetic properties of drugs by the following methods (i) coupling, (ii) conjugating, (iii) as fusion protein, or (iv) encapsulation with polymers.\textsuperscript{22} Furthermore, albumin is nontoxic, biodegradable, and nonimmunogenic in nature, which makes it a better candidate for using it as a drug carrier protein.\textsuperscript{23–25}

Conjugation of albumin with insulin using linker chemistry has been reported in this study. The free cysteine at the 34th position on albumin is a site most explored for the conjugation purpose both \textit{in vivo} and \textit{in vitro}.\textsuperscript{26–28} However, in this study, the surface lysine residues on albumin have been modified to create free thiols using linker chemistry. Here, we introduce an average of 6–8 thiols on albumin using 2-iminothiolane as reported in earlier studies.\textsuperscript{27,28} The ε-amine present in the B29 lysine residue of insulin was functionalized with the heterobifunctional linker (Mal-PEG\textsubscript{2}-NHS) to generate a free maleimide group. Later, it was made to react with the thiolated bovine serum albumin (BSA) to form insulin–albumin conjugates. The conjugated product was characterized for its physicochemical properties and studied for its glucose uptake activity. The glycemic control attained by the insulin–albumin conjugate was evaluated for 72 h in STZ-induced diabetic male Wistar rats in comparison with native insulin.

\section*{RESULTS AND DISCUSSION}

The primary objective of the study was to prolong the action of insulin by increasing its half-life that could potentially extend its normoglycemic effect in diabetics. The prime focus of the study was to develop a bioconjugate where albumin is conjugated with multiple insulin molecules. Modification of the ε-amines of the surface lysine residues using selective and specific linkers was implemented for both thiolation of BSA and addition of the heterobifunctional linker to insulin.

\textbf{Thiolation of BSA and Its Estimation.} Albumin is known to be a versatile drug carrier, and the only free sulphhydryl group available for modification is cysteine 34 residue and has been used extensively though its reactivity is limited.\textsuperscript{29} BSA is known to consist of 60 lysine residues\textsuperscript{30,31} and thus amine-specific modification was performed using 2-iminohistidine (2-IT) to create free thiols on the surface, which would be viable for cross-linking with insulin. 2-IT undergoes a ring opening reaction when it reacts with the free amines on BSA at pH 7.4.\textsuperscript{31} Optimization of thiol functionalization was performed at both 4 °C and room temperature (30 °C). When BSA was made to react with 20-fold excess of 2-IT at 4 °C, the thiolation rate was slow; however, an average of 6–8 thiols were generated in 2 h when the reaction was performed at room temperature (30 °C) (Figure S4). The estimation of thiol was performed using the 4,4′-dipyridyl disulphide (4-PDS) method, which is known to give a very precise value for free –SH by absorbing strongly at 324 nm when compared to the DNTB method at pH 7.4.\textsuperscript{32} However, it has to be noted that the sites of thiolation are plausible sites and have not been confirmed in this study (Figure S6).

\textbf{Addition of the Heterobifunctional Linker (Mal-PEG\textsubscript{2}-NHS) to Insulin.} Insulin, on the other hand, was treated with the heterobifunctional linker maleimide-PEG\textsubscript{2}-N-hydroxysuccinimide (Mal-PEG\textsubscript{2}-NHS), where the latter undergoes a nucleophilic substitution reaction with lysine B29 of insulin to form insulin-PEG\textsubscript{2}-MAL. The reaction was performed in physiological pH 7.4, where the pK\textsubscript{a} of lysine is lower in insulin.\textsuperscript{33} Thus, at the physiological pH, lysine would react more actively compared to N-terminal amine groups in insulin.\textsuperscript{34} The similar chemistry with the heterobifunctional linker having a longer PEG chain (MAL-PEG-NHS, \(M_w 5000\) Da) has been used in the modification of exendin.\textsuperscript{35} The product insulin-PEG\textsubscript{2}-MAL was later allowed to react with the thiolated BSA to form insulin–albumin conjugates.

\textbf{Bioconjugation of Insulin to Albumin.} The maleimide group on the insulin reacted specifically with the highly

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\textbf{Figure 1.} Scheme for the synthesis of the insulin–albumin conjugate.
reactive sulphydryl groups on BSA to form irreversible thioether linkages (Figure 1). Optimization of the reactions was performed where BSA-2IT (0.25 mM) was allowed to react with different molar concentrations of insulin-PEG2-MAL (ratios 1:8, 1:12, 1:16) (Table S1). Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was run to check if the conjugation was successful (Figure S7). It was observed that BSA-2IT (0.25 mM) and insulin-PEG2-MAL (2 mM, 8-fold excess) (ratio 1:8) gave the best results without any residual insulin. The optimized reaction ratio (1:8) was used as the final reaction ratio, and the conjugate was later purified and characterized.

**Purification and Characterization of the Conjugate.**

Once the reaction was complete, the product was purified using size exclusion chromatography which revealed that the bioconjugate elutes out at 35 min, which is before the elution time of BSA (hinting toward a molecular weight above BSA) (Figure 2A). The peaks were collected, and a reducing SDS-PAGE gel was run which showed a band above BSA, confirming the bioconjugation of insulin to albumin (Figure 2B). Fluorescence spectroscopy measurements suggested that there was quenching of the signal, at emission (280 nm) and excitation (345 nm) when compared to BSA (Figure 2C,D). This was due to the reduction in the signal of tryptophan of BSA owing to the covering of the albumin surface by conjugated insulin molecules. The molecular radius and hydrodynamic volume of the bioconjugate was compared with BSA, and it was found that both the molecular radius (8.6 to 57.9 nm) and hydrodynamic volume (332.8 to 77,472.1) had increased in case of the bioconjugate (Table 1). The bioconjugation is generally known to increase the hydrodynamic volume of the bioconjugates to a greater level along with the slight increase in molecular weight, and thus it is expected to reduce renal clearance and proteolytic degradation.24

**Glucose Uptake Assay.** The activity of insulin was one of the concerns after bioconjugation; hence we evaluated it in vitro in 3LT3-L1 cell lines (mouse embryo fibroblast cells) for the uptake of glucose. The cells were first incubated with 2-NBDG which is a fluorescent analogue of glucose. Later, the cells were treated with the same concentration (5 μM) of insulin and bioconjugate, and the study was performed in triplicates. After 1.5 h of incubation, the relative mean fluorescence intensity (MFI) of 2-NBDG taken up by the cells treated with insulin and bioconjugate was measured. The relative MFI was compared, and it was observed that the bioconjugate had an MFI of 16.16 compared to insulin which had an MFI of 19.42. This indicated that the bioconjugate had similar glucose uptake activity as compared to insulin (Figure 3). There was no visible toxicity/cell death observed in vitro in the cell lines after treatment with insulin or bioconjugate (Figure S8). These results were encouraging and led to the further examination on the efficacy of the bioconjugate in an animal model.

![Figure 2. Characterization of the insulin–albumin conjugate. (A) Size exclusion chromatography of BSA, insulin, and the bioconjugate. (B) SDS-PAGE (8%) gel represents the bands of the bioconjugate above BSA where lane 1: protein marker, lane 2: bioconjugate, lane 3: BSA, and lane 4: insulin. (C) Emission spectra of BSA and the bioconjugate. (D) Excitation spectra of BSA and the bioconjugate.](image)

| sample             | mean diameter (d, nm) | mean radius (r, nm) | hydrodynamic volume (4πr³/3) | theoretical molecular weight (approx.) |
|--------------------|-----------------------|---------------------|-----------------------------|---------------------------------------|
| BSA                | 8.6                   | 4.3                 | 332.8                       | 133 kDa                               |
| bioconjugate       | 52.9                  | 26.45               | 77,472.1                    | 207–232 kDa                           |
Evaluation of the Glucose Lowering Pattern in Male Wistar Rats. The use of STZ to induce type-1 diabetes in rats has been immensely popular because of its ease of use. The previous established literature showed a dosage of 60 mg/kg body weight of STZ to induce diabetes in Wistar rats with glucose levels reaching around 500 mg/dL. In this study, induction of diabetes is reported at a dose of 50 mg/kg body weight for male Wistar rats. The physical characteristics of STZ induction such as a stained tail with a grayish coat around the lower region of the body were quite evident. A total of four groups were used for the study: group 1: normal, group 2: diabetic control, group 3: insulin (5 IU/Kg), and group 4: bioconjugate (5 IU/Kg). There was a minimum of 250 mg/dL increase from the normal value of the blood glucose levels observed in the animals treated with STZ. A duration of 24 h was sufficient to observe this increased blood glucose levels in STZ-treated animals, and the values remained persistently high throughout the study period in the diabetic control group. A drastic fall in the body weights was observed in the diabetes control group, and gradual restoration was observed in insulin and bioconjugate treated (5 IU/kg) groups.

An average increase of 250 mg/dL in the blood glucose of the STZ-treated group was considered for the comparison with other groups for their change in blood glucose. In the group treated with insulin (5 IU/kg), a remarkable decrease in blood glucose by an average of 315.8 mg/dL in the first hour was observed. However, these reduced glucose levels were not maintained, and a drastic increase of blood glucose levels was observed.
observed after 3 h and remained elevated at an average of 426 mg/dL after 24 h of treatment. However, in the bioconjugate-treated group (5 IU/kg), there was a reduction of an average of 193.2 mg/dL after the 1st hour of treatment, an average reduction of 274 mg/dL after 3 h, and the blood glucose levels were reduced by 234 mg/dL up to 72 h of treatment (Figure 4) without death of any animal due to hypoglycemic shock.

Thus, the results show a clear-cut indication of diabetes induction in all the animals. The Tukey’s multiple comparison test depicted that the P value ranged from 0.04–0.8 in time interval (0 to 72 h) ANOVA analysis. The variation in the P value is due to high standard deviation, and thus the mean difference plot of insulin (5 IU/kg) with the bioconjugate (5 IU/kg) treated groups was plotted. The graph represents that the bioconjugate was capable of controlling the blood glucose levels when compared to insulin up to 72 h with a minimum mean difference of 60 mg/dL (Figure 5A). The plasma concentration was tested after 24 h of treatment for both bioconjugate (5 IU) and insulin (5 IU) groups and was compared to the normal group. The results observed in the experiment represent that there was almost 3-fold excess of plasma insulin concentration in the bioconjugate treated group (mean = 142 μIU/mL) when compared to the insulin treated group (mean = 52 μIU/mL) (Figure 5B).

The breakdown of micro-anatomical features including necrotic changes, β-cell degranulation, pycnotic β-cell nuclei, and severe vacuolation is indicative of histological changes observed after STZ treatment.38 In this study, degranulation of β-cells in the pancreas, mild congestion, and inflammation in the liver and kidney in all STZ-treated groups have been observed (black Arrows shown in Figure 6). There were no necrotic changes observed in the insulin and bioconjugate treated groups, which reveals that there was no toxicity observed due to the treatment of insulin and the bioconjugate.

These results are indicative that the bioconjugate effectively prolonged the hypoglycemic effect for a period of at least 72 h. Similar chemistry can be used to conjugate insulin with human serum albumin (HSA). The conjugate thus produced can be studied to establish treatment protocols for pre-clinical trials.

### CONCLUSIONS

In this study, we present an insulin–albumin conjugate where two proteins are cross-linked using linker chemistry. Thiolated BSA is conjugated to maleimide functionalized insulin to produce insulin–albumin conjugates. Conjugation of albumin to insulin increases the molecular weight of insulin and reduces renal clearance thus, extending its half-life. Also, the heterobifunctional linker used is short in length compared to other studies resulting in possible decrease in the proteolytic degradation due to steric hindrance. This is the first report on conjugating multiple insulin molecules to albumin which is known to have prolonged half-life in blood circulation to the best of the authors’ knowledge. The insulin–albumin conjugate has near-optimal activity of insulin with better glycemic control up to 72 h not only compared to native insulin but also to other reports on bioconjugation.16,17 Further experiments with larger data sets for animal studies would improve the understanding of the glycemic regulation of this bioconjugate. This insulin–BSA conjugate could be a proof of concept to develop the insulin–HSA conjugate which could be a potential lead molecule for treatment of diabetes and also can be used in combination with the existing analogues of insulin.

### EXPERIMENTAL PROCEDURES

**Chemicals.** Human recombinant insulin, Traut’s Reagent (2-iminothiolane, HCl), and Aldithiol were procured from Sigma-Aldrich, India. Maleimide-PEG2-N-hydroxysuccinimidyl ester (Tokyo Chemical Industry, product number: M3079), BSA fraction V, and streptozotocin were obtained from Sisco Research Laboratories Pvt. Ltd. (SRL)—India. All other chemicals and reagents used in this study were of analytical or molecular biology grade. A human insulin ELISA kit was procured from RayBiotech Life, Inc. All reactions were performed using phosphate buffer saline (PBS) pH 7.4.

**Thiolation of BSA and Its Estimation (Step 1).** BSA (BSA-0.25) was reacted with 2-iminothiolane (2-IT)36,39 at different molar ratios, and the thiols generated on BSA were estimated using aldrithol-4 (4-PDS)40 at different time intervals of 0, 0.5, 1, 2, 3, 4, and 12 h (Figure S3). Thiols...
were generated on albumin by modifying the methods previously described elsewhere.27,28 Number of thiols per BSA (SH/BSA) was calculated using the molar extinction coefficient of 4-thiopyridine (4-TP), that is, 1.98 × 10^4 M^-1 cm^-1. The generation of thiols/BSA was standardized using different ratios of BSA and 2-IT (Figures S3 and S5). Finally, BSA (0.5 mM) was treated with 2-IT (10 mM, 20-fold excess) for 2 h at 30 °C, and it generated an average of 6–8 thiols per BSA molecule. Following the reaction, excess 2-IT was removed using a centrifugal concentrator with a 3 KDa cut off membrane filter where the buffer was added two times and was later concentrated for further bioconjugation reactions.

**Addition of a Heterobifunctional Linker (Mal-PEG2-NHS) to Insulin (Step 2).** Maleimide-PEG2-N-hydroxysuccinimidy l ester (Mal-PEG2-NHS) was used as the linker to generate free maleimides on the lysine B29 residue of insulin. A similar heterobifunctional linker (Mal-PEG2-NHS)-5 KDa with a longer PEG chain was used for conjugation of exendin-4. Here, insulin (0.5 mM) was treated with 10-fold-excess of Mal-PEG2-NHS (5 mM) for 2 h in 30 °C. After the reaction was complete, the excess of Mal-PEG2-NHS was removed using a centrifugal concentrator with a 3 KDa cut off membrane filter. Thus, a free maleimide group generated on insulin would further react with the free thiols on BSA. The column was equilibrated with PBS (1×) buffer, pH 7.2 at 1 mL/min flow rate. The final reaction mixture was loaded through the loading pump and eluted with one column volume of PBS and peaks were collected using a fraction collector. The pooled fractions of each peak were concentrated for further bioconjugation reactions.

**Bioconjugation of Insulin and BSA (Step 3).** Optimization of insulin–albumin conjugates was performed by reacting thiolated BSA (step 1 product) with different molar concentrations of insulin-PEG-Mal (BSA-2IT (0.25 mM): Ins-PEG2-Mal ratios of 1:8, 1:12, and 1:16) (step 2 product) at different temperatures (at 4 °C and at room temperature ~ 30 °C). The size of each product was verified by SDS-PAGE. The final reaction ratio was selected, where BSA-SH (0.25 mM) reacted with Insulin-PEG-Mal (2 mM, 8-fold excess) for 2 h at room temperature (30 °C). Further, the reaction was completed by incubating it overnight at 4 °C to form insulin–albumin conjugates.

**Purification of the Bioconjugates.** The bioconjugate was purified using a size exclusion column Superdex 75pg, 16/600 with AKTA start FPLC system (GE healthcare life sciences). The column was equilibrated with PBS (1×) buffer, pH 7.2 at 1 mL/min flow rate. The final reaction mixture was loaded through the loading pump and eluted with one column volume of PBS and peaks were collected using a fraction collector. The pooled fractions of each peak were concentrated for further characterizations.

**Characterization of the Bioconjugate.** Size of the modified bioconjugate was determined using SDS-PAGE run in the Bio-Rad Electrophoresis Unit. The concentrated insulin–albumin conjugate was subjected to SDS-PAGE (8%) and a constant current of 120 V was applied to the gel. The size of the bioconjugate was compared with a known standard marker.

**Fluorescence Measurements.** The effect of bioconjugation on fluorescence of albumin was measured at 0.5 mg/mL concentration. Both bioconjugate and BSA were compared for fluorescence emission at 280 nm and excitation at 345 nm. The fluorescence was measured using the instrument Agilent Cary eclipse spectrophotometer.

**Dynamic Light Scattering.** The hydrodynamic volume of the bioconjugate and BSA with concentration (0.5 mM) was measured using Brookhaven Zeta Pals with a 15 mW solid state laser at a wavelength of 658 nm having a fixed scattering angle of 90°.
were followed while handling and conducting experiments with animals.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c06119.

Glutathione standard for thiol estimation by 4-PDS, optimization of thiolation of BSA, optimization of the bioconjugation reaction, sites of modification, glucose uptake assay-cell toxicity after treatment, and animal grouping information (PDF)

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**Author Contributions**

S.S.R. and A.K. together conceptualized the problem. S.S.R. performed the experiments and characterizations. Y.S. guided S.S.R. for the animal studies performed in St. Aloysius College, Mangaluru. S.S.R., Y.S., and A.K. together evaluated the experimental results and wrote the manuscript.

**Notes**

The authors declare no competing financial interest.

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