Iranian Journal of Pharmaceutical Sciences
2024: 20 (3): 227- 241
https://journals.sbmu.ac.ir/IJPS

Original Article

Formulation Development and *in-vivo/in-vitro* Characterization of Novel Glucose Responsive Hydrogel for Insulin Delivery

Rupesh A. Saindane[a,c*] and Sharwaree R. Hardikar[b,c]

[a]Department of Pharmaceutics, MET Institute of Pharmacy, Nashik, MH, 422003 India, [b]Department of Pharmaceutics, SSWVSM's Tatyasaheb Kore College of Pharmacy, Warananagar, 416113 MH, India, [c]Department of Pharmaceutics, SGRS College of Pharmacy, Saswad Dist Pune MH, 412301 India.

Abstract

Glucose-responsive delivery systems intelligently regulate insulin release in response to fluctuating blood glucose levels, offering a more controlled approach to diabetes management. The current study introduces a novel glucose-responsive hydrogel system for enhanced insulin delivery. The hydrogel, formulated from chitosan and Poloxamer 407, exhibits unique thermo-responsive and pH-sensitive characteristics, making it suitable for subcutaneous insulin delivery. The research encompasses comprehensive in vitro and in vivo analyses to evaluate the formulation's efficacy and responsiveness to glucose concentrations. In vitro studies demonstrated that the hydrogel's solubilization rate varies with pH and glucose levels, crucial for controlled insulin release. Notably, the hydrogel exhibited an extended and more stable control of blood glucose levels compared to conventional insulin treatments. One significant finding is the hydrogel's rapid in vivo gelation and biodegradability, indicating its safety and effectiveness in a physiological environment. In vivo experiments conducted on diabetic rats showcased the hydrogel's glucose-responsive behavior. Moreover, the hydrogel's ability to modulate insulin release in response to changing glucose levels was distinct from traditional insulin therapies, highlighting its potential to manage multiple hyperglycemic episodes after a single dose. Overall, this research marks a significant advancement in insulin delivery systems. The development of this glucose-responsive hydrogel system presents a promising approach for achieving more controlled and stable blood glucose levels in individuals with diabetes, potentially enhancing their quality of life. The findings suggest opportunities for further optimization, particularly in insulin content, to tailor the system to the specific needs of the diabetic population.

Keywords: Glucose-responsive delivery; Insulin; pH-Sensitive hydrogels; Diabetes; Chitosan; Poloxamer.

Corresponding Author: Rupesh A. Saindane, Department of Pharmaceutics, M. E. T's Institute of Pharmacy, Nashik Bhujbal Knowledge City, Adgaon, Nasik-422003, Maharashtra, India. E-mail: rupeshsaindane@gmail.com

Cite this article as: Saindane RA, Hardikar SR. *Formulation Development and In-vivo/ In-vitro Characterization of Novel Glucose Responsive Hydrogel for Insulin Delivery*. J. Pharm. Sci., 2024, 20 (3): 227- 241.

DOI: https://doi.org/10.22037/ijps.v20i3.44381

1. Introduction

The World Health Organization defines diabetes mellitus as a chronic, metabolic disease characterized by elevated blood glucose levels leading to severe cardiac, ocular, renal, and neural complications [1]. Ranking among

the top four non-communicable diseases globally, diabetes has consistently risen in incidence and prevalence. Currently, about 537 million adults, or 10.5% of the population aged 20-79, are estimated to have diabetes, with projections indicating an increase to 11.3% (643 million) by 2030 and 12.2% (783 million) by 2045 [2].

Exogenous insulin therapy is crucial for managing hyperglycemia, especially in Type 1 diabetes, and is also used in advanced Type 2 diabetes cases. Despite its effectiveness, traditional subcutaneous insulin administration poses challenges like hypoglycemia, variable patient adherence, and potential hospitalization. Therefore, developing an insulin delivery system that can sense blood glucose levels and adjust insulin release is vital for optimal diabetes management [3–5].

Enzyme-based polymeric hydrogels, notably those using pH-sensitive polymers and glucose-reactive enzymes, have shown promise in overcoming the limitations of conventional insulin therapy [6] These systems utilize enzymes like glucose oxidase, which are solutions upon injection and form in-situ gels in response to physiological stimuli like temperature or pH. This enzyme converts glucose into gluconic acid, decreasing the microenvironment's pH and increasing insulin release in response to blood glucose concentration [7].

However, challenges exist, such as the oxygen dependency of the glucose-to-gluconic acid conversion and hydrogen peroxide's inhibition of glucose oxidase activity [8] Integrating an enzyme like catalase, which consumes hydrogen peroxide and generates oxygen, can enhance the functionality of glucose-responsive systems [9].

Chitosan, a biocompatible and biodegradable natural polymer, is extensively used in hydrogel delivery systems due to its pH-responsive solubility and high mechanical strength, making it suitable for glucose-responsive drug delivery [10, 11]. However, chitosan often requires adjuvant agents like Poloxamer to enhance its drug delivery capabilities [12–15]. Poloxamers, amphiphilic triblock copolymers, show temperature-dependent sol-gel transitions and are used to modulate in situ gel formation at body temperature [16].

In the current study, we developed a glucose-responsive insulin delivery system combining chitosan, poloxamer, glucose oxidase, and peroxidase to optimize diabetes management by addressing current therapy challenges.

2. Materials and Methods

2.1. Materials

Actrapid (Human Insulin) 40IU/ml Solution for Injection, manufactured by Novo Nordisk India Pvt Ltd., was acquired from a local pharmacy. Poloxamer 407 (Lutrol MC-127) was kindly provided as a gift sample by BASF India. Chitosan, with a 90% degree of deacetylation, was purchased from Sisco Research Lab, Mumbai. All other chemicals used were of analytical grade, ensuring the reliability and accuracy of the experimental procedures.

2.2. Optimization of Chitosan and Poloxamer Concentrations and Preparation of Glucose Responsive Hydrogels

A pH and temperature-responsive hydrogel system was developed by optimizing concentrations of chitosan (20-26% w/w) and

Poloxamer 407 (1.5-2% w/w). The formulations were prepared by modifying the cold method reported earlier [17] Briefly, chitosan was dissolved in 1.0% acetic acid to form Solution A, which was sterilized and stored under refrigeration. Solution B, consisting of Poloxamer 407, was dispersed in water, refrigerated for 72 hours, sterilized, and then supplemented aseptically with glucose oxidase and peroxidase enzymes. The two solutions were then mixed gently and aseptically. The final formulation was completed by aseptically adding 50IU of insulin. Placebo hydrogels and Chitosan-Poloxamer (CS-PO) Hydrogels without Insulin, glucose oxidase, or peroxidase were prepared using the same methodology.

2.3. Physical Characterization

The final formulation, placebo hydrogel, and CS-PO hydrogel were assessed visually for their physical appearance and pH.

2.4. Thermal Characterization

Thermal properties of the Chitosan solution, Poloxamer solution, final formulation, placebo hydrogels, and CS-PO hydrogels were analyzed using Differential Scanning Calorimetry. Samples in aluminum crucibles were heated from 30-350°C at a rate of 10°C/min in a nitrogen atmosphere, with the results analyzed using STAR^e 9.20 Software.

2.5. In Situ Sol-Gel Transition Properties

The gelling temperature of the Poloxamer Solution, final formulation, placebo hydrogel, and CS-PO hydrogel were recorded using the Vial tilting method [18] A fast vial flip method determined the gelling time and the effect of temperature on the gelling time. Briefly, sol was exposed to different temperatures in a vial, and the time at which it ceased to flow was noted as the gelling time[19] This research focuses on subcutaneous insulin delivery, necessitating that the hydrogels be compatible with clinical needles. An injectability study assessed the feasibility of delivering hydrogels through commonly used 23G and 26G needles.

2.6. Solubility Study

pH-dependent and glucose-dependent solubility studies were performed on the CS-PO polymeric system and optimized formulation. Solubilization rates were calculated as per **Equation**:

$$\text{Solubilization} = \frac{\text{Initial weight} - \text{weight at time 't'}}{\text{Initial weight}} \times 100$$

2.7. In Vitro Release Profile

The in vitro drug release from the final formulation at different glucose concentrations was determined as a function of time. [20] 0.5 ml of the final formulation was allowed to gel at 37°C. The resulting gel was placed in 5 ml phosphate buffer pH 7.4 in an orbital shaking incubator at 37°C at 70 rpm. Drug release was determined at different glucose concentrations, viz. 100, 300, 500 mg/dl. The amount of Insulin released was determined by the Biuret method on a UV-visible spectrophotometer at 546 nm.

In another study, the ability of the formulation to release insulin as a response to the changing glucose concentration was evaluated by monitoring insulin release at fluctuating glucose levels. The study was designed similarly to the in vitro drug release. However, 1 mL of gel was taken and exposed to 5ml of phosphate buffer pH 7.4 containing 100 mg/dl, 300mg/dl, or 500 mg/dl glucose. The gel was exposed to one buffer for a specific time, and

then it was removed from that buffer and exposed to a buffer with a different glucose concentration. The amount of insulin released was determined at each point in time.

2.8. In vivo Gelling Behavior

An in vivo gelation study was performed on Sprague Dawley rats, assessing the occurrence of swelling at the injection site and the presence of gel residue post-swelling. Once the swelling at the injection site subsided, the animals were euthanized. The injection site was dissected to check for any residual gel and signs of tissue damage, and biodegradability and biocompatibility were confirmed.

2.9. Induction of Diabetes in Rat models

The experimental protocol followed the Organization for Economic Cooperation and Development (OECD) guidelines [21] All animal experiments followed institutional guidelines, and the Committee approved the procedure for Control and Supervision of Experiments on Animals (CPCSEA). Efforts were made to minimize the number of animals used for experimentation.

A total of 16 male Sprague-Dawley rats were used in this study. The rats were housed in a controlled environment with a 12-hour light/dark cycle at a temperature not exceeding 25°C, with free access to laboratory chow and water.

Diabetes was induced in rats by the method described by Kim et al. [22]. The test animals were administered a daily dose of intraperitoneal 120 mg/kg Alloxan dissolved in distilled water. The rats' blood sugar levels were measured using a glucometer to confirm the induction of rats. Rats with blood glucose levels above 250 mg/dL were considered diabetic and included in the study after a week of stabilization.

2.10. Comparative in-vivo efficacy in diabetic Rats

The comparative in-vivo efficacy of the study formulation against a placebo and a conventional insulin formulation was evaluated in diabetic rats. The rats were divided into four groups (n=4 per group), each receiving a different formulation. The non-diabetic control group received an equivalent volume of the vehicle. In contrast, diabetic groups received a placebo formulation, commercial conventional insulin formulation, or the Glucose Responsive Hydrogel subcutaneously. as outlined in **Table 1**.

An oral glucose dose of 2 g/kg was administered to all animals at 0- and 6 hours post-gel administration. Blood samples (500 µl) were taken from the tail vein at specified intervals (0, 0.5, 1, 2, 4, 6, 8, 12, and 24 hours). Following each sampling, the rats were given 1 ml of normal saline to maintain stable central compartment volume and electrolyte balance. Blood glucose levels were measured using a glucometer, and the results were analyzed to compare the efficacy of the formulations based on blood glucose level variations.

Table 1. Comparative Study Groups for Evaluating Efficacy of Different Formulations in Diabetic Rats**.**

Treatment Group	Description	Treatment Formulation	Dose
Control	Healthy rats receiving normal saline	Normal Saline	0.2 ml
Placebo	Diabetic rat receiving Placebo	Blank CS-PO gel without Insulin, GOD, and POD	0.2 ml
1	Diabetic rats receiving marketed formulation	Commercial Insulin (Actrapid Human Insulin)	4 IU/kg
2	Diabetic rat receiving test formulation	Glucose Responsive Hydrogel (Final Formulation)	4 IU/kg

3. Results and Discussion

3.1. Optimization of Chitosan and Poloxamer Concentrations and Preparation of Glucose Responsive Hydrogels

Developing the CS-PO hydrogel system required a careful optimization of the concentrations of its two main components, Chitosan and Poloxamer 407, to achieve the desired sol-gel transition properties conducive for glucose-responsive delivery.

The initial experiments involved testing a range of Chitosan and Poloxamer 407 concentrations. While some formulations (F1, F2, F5, and F6) did not form gel up to 50°C, others (F3, F4, F7, and F8) demonstrated gelling at room temperature within 5 to 15 minutes. However, these initial formulations did not exhibit ideal characteristics for glucose-responsive release.

Based on the initial findings, further optimization was pursued. Formulations F13 and F14, with lower Chitosan concentrations and slightly varied Poloxamer 407 levels, showed promising sol-gel transitions at 32°C within 6 to 8 minutes. These formulations suggested a more effective concentration range for the desired hydrogel properties.

Subsequent trials led us to the formulation comprising 1.5% Chitosan and 20.5% Poloxamer 407, which exhibited gel formation in 5.38 minutes at 32°C. This concentration was determined to be optimal for the development of our glucose-responsive CS-PO hydrogel system.

These findings underpin the critical role of precise polymer concentration in developing an effective CS-PO hydrogel system, with implications for its use in glucose-responsive drug delivery applications. The details of formulation compositions are summarized in **Table 2**.

Table 2. Optimization Chitosan and Poloxamer 407 Concentrations**.**

Formulation Code	Chitosan Concentration (%)	Poloxamer 407 Concentration (%)	Observation
Initial Screening			
F1	2.0	20	No gelling up to 50°C
F2	2.0	22	No gelling up to 50°C
F3	2.0	24	Gelled at room temp within 10 minutes
F4	2.0	26	Gelled at room temp within 5 minutes
F5	1.5	18	No gelling up to 50°C
F6	1.5	20	No gelling up to 50°C
F7	1.5	21	Gelled at room temp within 15 minutes
F8	1.5	22	Gelled at room temp within 10 minutes
Optimization Phase			
F9	2.0	22.5	No gelling up to 50°C
F10	2.0	23	Gels at room temp within 15 minutes
F11	2.0	23.5	Gels at room temp within 10 minutes
F12	1.5	20.2	Gels at 40°C
F13	1.5	20.4	Gels at 32°C within 8 minutes
F14	1.5	20.6	Gels at 32°C within 6 minutes
F15	1.5	20.8	Gelled at room temp within 10 minutes
Optimized Formulation			
Optimal	1.5	20.5	Gels in 5.38 minutes at 32°C

3.2. Physical Characterization

All the hydrogel systems existed as a transparent and viscous solution at room temperature. The CS-PO hydrogel was a yellow-colored solution attributable to the presence of chitosan. In comparison, the Placebo hydrogel and Final formulation appeared reddish-brown colored. The change in appearance can be attributed to the reddish color of the peroxidase enzyme.

The pH of the 1% acetic acid solution was 2.80. Solubilization of chitosan in acetic acid increased pH up to 4.58. Adding Poloxamer 407 to the formulation did not alter the pH significantly. However, the incorporation of glucose oxidase (GOD) and peroxidase (POD) caused a slight increase in pH up to 4.69. The pH of the formulation was recorded at 4.74.

3.3. Thermal characterization

The Differential Scanning Calorimetry (DSC) thermograms of the Chitosan and Poloxamer 407 solutions, CS-PO hydrogel, and the optimized formulation are presented in **Figure 1**.

The DSC thermogram of the Chitosan solution exhibited a broad endothermic peak at approximately 108°C, while the Poloxamer 407 solution showed a similar peak at 119°C. These endothermic peaks are characteristic of the evaporation of free and bound water from the polymer solutions.

In polymeric systems, water exists in two forms: free water, which evaporates at temperatures comparable to bulk water, and bound water, which is attached to polymers due to hydrophilic interactions and evaporates at higher temperatures [23]

For the CS-PO hydrogel, a sharp endothermic peak was observed at 105°C, indicating the presence of predominantly free water. The final formulation displayed a broader endotherm at 107°C, suggesting the absence of significant thermal or chemical changes during the gelling process.

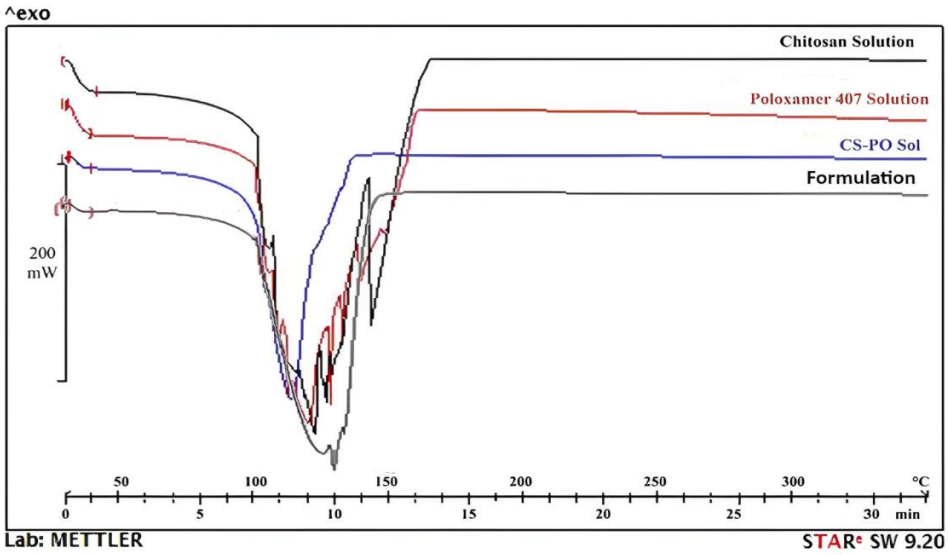

Figure 1. DSC thermograms of polymeric solutions, blank hydrogel, and optimized formulation.

Compared to the individual polymers, the sharper endotherm in the CS-PO hydrogel indicates that only free water evaporates, leaving the bound water intact within the hydrogel. This observation implies that the hydrogel system retains more bound water than the individual polymer components. The presence of water in the hydrogel is crucial for maintaining its integrity and solubility, facilitating the diffusion of entrapped molecules, and contributing to the structure of water-filled pores [23]

Furthermore, the thermogram of the final formulation shows a peak with higher enthalpy, which is attributed to the unfolding of enzymes at elevated temperatures. This peak provides valuable insight into the thermal behavior of the enzymes within the hydrogel system [23–25]

3.4. In Situ Sol-Gel Transition Properties

3.4.1. Gelling Temperature

The temperature-dependent gelling of the current hydrogel system is due to Poloxamer 407. Thus, the poloxamer solution gelled at the lowest temperature compared to the CS-PO gel and Formulation. The temperature-dependent gelling behavior of Poloxamer stems from its Lower Critical Solution Temperature (LCST), where hydrophilic polymer-solvent interactions are surpassed by hydrophobic polymer-polymer interactions above a certain temperature [26] However, adding Chitosan intensifies these hydrophilic interactions through hydrogen bonding, necessitating greater thermal energy to disrupt these bonds. Consequently, the CS-PO hydrogel exhibits a higher gelling temperature than the Poloxamer solution alone. The gelling temperature of the CS-PO hydrogel, as outlined in **Figure 2A**, showed a notable increase compared to Poloxamer 407 solutions. This elevated sol-gel transition temperature is linked to the synergistic interaction between Chitosan and Poloxamer 407, stabilizing the polymers in solution at higher temperatures. Adding insulin, GOD, and POD did not impact gelling behavior.

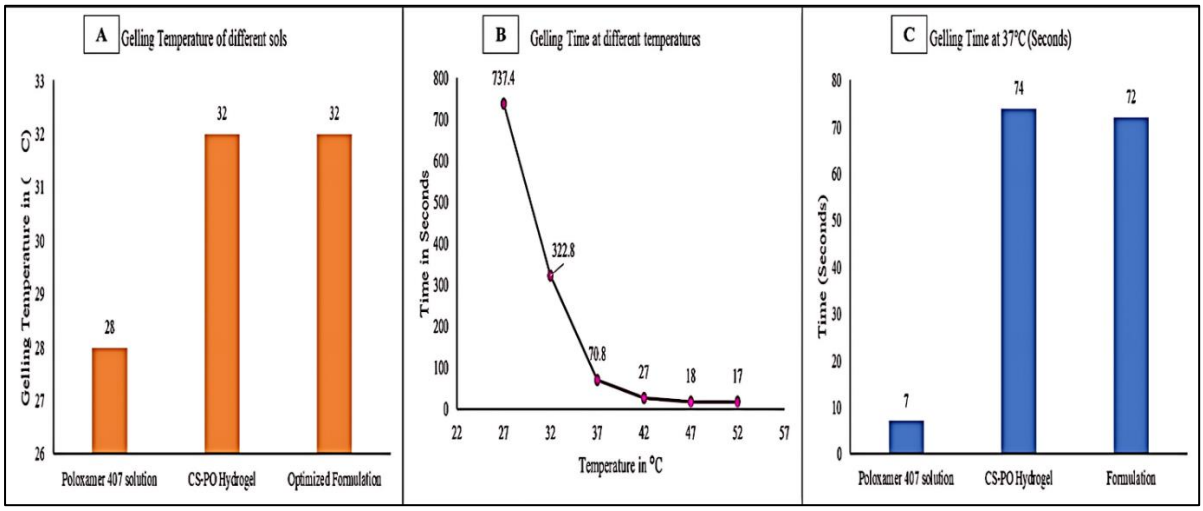

Figure 2. In situ sol-gel transition properties for hydrogel systems (A) Gelling Temperature (B) Effect of temperature on gelling time (C) Gelling time at 37°C.

3.4.2. Gelling Time

A temperature-dependent gelation study was conducted to understand the gelling time and effects of temperature on the gelation process. At 22°C, no gelling occurred, but as the temperature rose beyond 27°C, gelation occurred more rapidly. However, beyond 47°C, further temperature increases did not affect the gelation time. The micellar nature of Poloxamer can be attributed to this behavior. In aqueous solutions, Poloxamers form micelles and remain soluble due to hydrogen bonding between water and the hydrophilic part of the polymer.

Heating the solution disrupts these bonds, leading to increased hydrophobic interactions and gel formation [27] At 22°C, the heat was insufficient to break the hydrophilic interactions. Above 27°C, these interactions began to break, but not all at once, requiring more time for complete gelation. At 47°C, the rate of bond breakage peaked, resulting in rapid gelling. Beyond this temperature, an increase in heat did not further accelerate gelation. The study's findings on temperature versus gelling time are illustrated in **Figure 2B**.

Given the intended subcutaneous application of the hydrogel, understanding its gelling time at body temperature (37°C) was essential. An excessively long gelling time could lead to burst drug release, while too rapid gelation might cause needle clogging. The comparison of the gelling times of Poloxamer 407 solution, CS-PO Hydrogel, and Optimized Formulation at 37°C are depicted in **Figure 2C**.

The Poloxamer 407 solution gelled rapidly at 37°C. In contrast, the CS-PO and formulation had similar gelling times, remaining in solution for over a minute, which was sufficient time for injection post-needle insertion. Thus, the study formulation demonstrated an adequate gelling time for in vivo administration.

3.4.3. Injectability

The study revealed that hydrogel could be effectively administered using both needle sizes. However, differences in the delivery pattern were observed due to the smaller diameter of the 26G needle compared to the 23G. When passed through the 23G needle, the hydrogel was dispensed in a continuous stream, whereas it was released in droplets with the 26G needle. Despite these variations in flow patterns, both needle sizes allowed for the smooth and complete delivery of the hydrogel. **Figure 3** illustrates the injectability of the CS-PO hydrogels using 23G and 26G needles, visually demonstrating the differences in flow patterns while underscoring the effective delivery through both needle types.

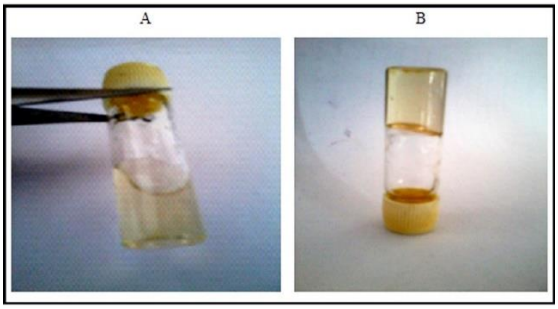

Figure 3. Sol-gel behavior of CS-PO Gel (A) Sol with fluid meniscus (B) Gel at elevated temperature.

3.5. Solubility Study

3.5.1. pH-dependent solubilization:

While the CS-PO solution demonstrated effective thermo-responsive properties, its pH-sensitive behavior is another essential aspect of a glucose-responsive delivery system.

The solubilization rate of the CS-PO hydrogel in response to different pH levels is presented in **Figure 4A.**

The study confirmed the pH-responsive nature of the hydrogel system. The solubilization process was rapid at pH 5.8, moderately fast at pH 6.8, and slowest at pH 7.4. This increased rate of solubilization at acidic pH levels is likely due to the enhanced solubility of Chitosan under such conditions. Therefore, the CS-PO hydrogel system, comprising Chitosan and Poloxamer, exhibits both pH and temperature responsiveness. This pH-dependent solubilization characteristic was leveraged to transform the system into a Glucose-Responsive Delivery System.

Figure 4A illustrates the comparative solubilization rates of the CS-PO hydrogel in phosphate buffers at pH 7.4, 6.8, and 5.8 over various time intervals, visually depicting its pH-sensitive dissolution behavior.

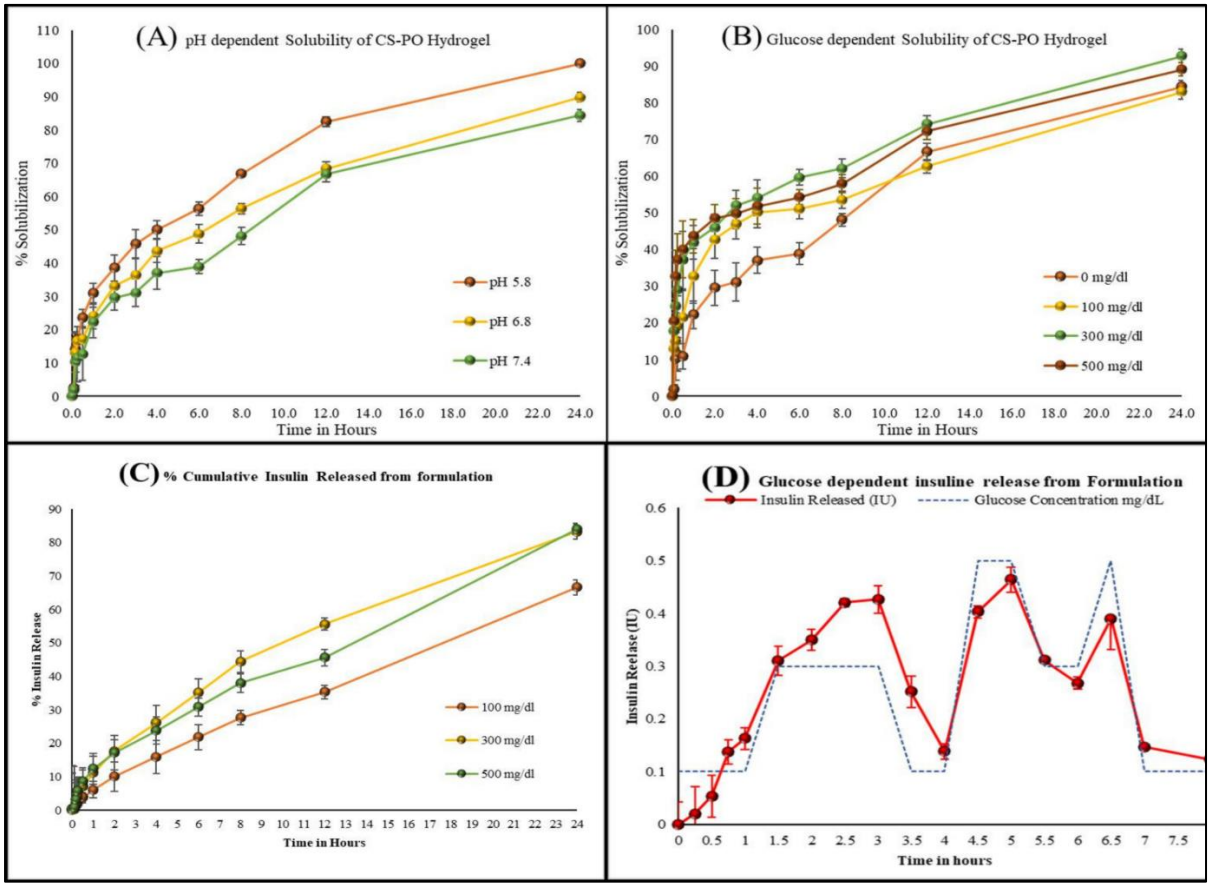

Figure 4. Solubility study of CS-PO hydrogel and in vitro release study of the formulation. (A) pH-dependent solubility, (B) glucose-dependent solubility, (C) insulin release at constant glucose concentrations, and (D) insulin release at fluctuating glucose concentrations.

3.5.2. Glucose-Dependent Solubilization

This study aimed to evaluate whether glucose concentration influences the solubility rate of the hydrogel at a constant pH and temperature. The findings revealed that solubilization rates increased with rising glucose concentrations. Initially, solubilization was directly proportional to the glucose concentration. However, no significant differences in solubilization rates were noted after one hour between glucose concentrations of 500 mg/dl and 300 mg/dl. This trend continued over three hours, with 500 mg/dl, 300 mg/dl, and 100 mg/dl glucose concentrations showing similar solubilization rates. Beyond twelve hours, glucose concentration ceased to have a discernible impact on solubilization.

The observed decrease in solubilization rate is attributed to enzyme inhibition by excess substrate, a well-known phenomenon in enzymatic reactions [28] In this context, high glucose levels likely inhibited Glucose Oxidase activity. Additionally, the accumulation of hydrogen peroxide, produced from the conversion of glucose to gluconic acid, further inhibited Peroxidase activity and reduced oxygen formation. Consequently, the inhibited action of both enzymes and the diminished oxygen concentration led to a decreased rate and extent of glucose-gluconic acid conversion, equalizing the solubilization rates across all glucose levels. The results of this study are represented in **Figure 4B.**

3.6. In Vitro Release Profile

3.6.1. In Vitro Release at Constant Glucose Concentration

The in vitro drug release from the formulation correlates with glucose levels of the medium. It exhibits increased insulin release with rising glucose concentrations, stabilizing continuously after 4 hours. This plateau is critical, indicating steady drug release without fluctuations in glucose.

The percentage of cumulative drug release at various glucose concentrations, as shown in **Figure 4C,** demonstrates the system's ability to augment insulin release in response to increased glucose levels. Notably, insulin release at 500 mg/dl glucose was lower than at 300 mg/dl, aligning with the findings from the glucose-dependent solubilization study and suggesting enzyme inhibition by excess substrate. However, this in vitro enzyme inhibition is unlikely to be a concern in vivo, as released insulin would reduce blood glucose levels, thus preventing enzyme inhibition.

3.6.2. In Vitro Glucose-Responsive Insulin Release

The in vitro glucose-responsive release test showed that insulin release from the formulation adjusts in response to varying glucose levels. An increase in glucose concentration led to heightened insulin release, whereas a decrease caused a reduction in release. This outcome differs from previous in vitro release studies.

Initially, exposure to 100 mg/dl glucose triggered insulin release within 15 minutes, which intensified with increasing glucose concentration to 300 mg/dl. Even after reaching a plateau stage, the release rate remained higher than at the lower glucose concentration. A corresponding decrease in insulin release was observed when the glucose level was reduced back to 100 mg/dl after 3 hours.

A subsequent increase to 500 mg/dl glucose increased the insulin release rate to higher

levels. The formulation maintained a glucose-responsive release pattern throughout the study. However, the insulin released was lower later for the same glucose concentration, likely due to the diminishing insulin content in the reservoir-based system. The graphical representation of this glucose-responsive release is depicted in **Figure 4D.**

3.7. In vivo Gelling Behavior

Following the subcutaneous (SC) injection of CS-PO hydrogel in male Sprague-Dawley (SD) rats, swelling was observed under the skin within 2 minutes, indicating a rapid sol-gel transition in vivo. When these rats were subsequently sacrificed and the injection site dissected, a firm gel was discovered beneath the skin, confirming the in-situ gelling properties of the formulation.

The biodegradability of the CS-PO gel was also a critical focus of the study. This aspect was evaluated using the same rats from the in vivo gelling study. The disappearance of swelling at the injection site suggested the hydrogel's elimination from the body. Within 24 hours of post-injection, the swelling had subsided. Following this observation, the animals were sacrificed, and the injection sites were examined. The absence of gel residue at the injection sites confirmed the complete biodegradability of the CS-PO gel. Moreover, no lesions or other signs of injury were noted at the administration sites. This lack of adverse effects further underscores the safety of the hydrogel for in vivo applications.

3.8. Comparative in-vivo efficacy in diabetic Rats

The in vivo studies on diabetic rats demonstrated the glucose-responsive behavior of the study formulation. The blood glucose levels of the control group, placebo group, Treatment Group-1 (receiving conventional insulin), and Treatment Group-2 (receiving the glucose-responsive formulation) are summarized in **Table 3** and **Figure 5**.

Table 3. Blood Glucose Levels for different groups during comparative in-vivo efficacy in diabetic Rats**.**

Sampling Time (Hours)	Average Blood Glucose Levels (n=4)			
	Control Group	Placebo Group	Treatment Group 1	Treatment Group 2
0	72	349	443	386
0.5	130	600	458	481
1	79	536	259	459
2	132	485	71	319
4	122	463	84	207
6	116	435	254	107
8	117	600	457	443
10	112	600	600	485
12	118	600	600	134

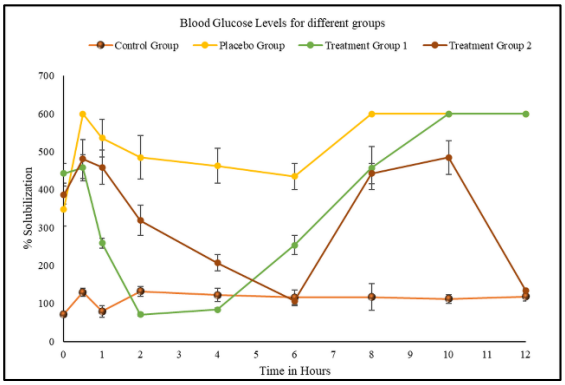

Figure 5. Blood Glucose Levels for different groups during comparative in-vivo efficacy in diabetic Rats.

Control Group: Blood glucose levels remained stable after administering 2 g/kg oral glucose doses at 0 and 6-hour intervals, showing homeostasis in non-diabetic rats.

Placebo Group: The diabetic rats receiving placebo hydrogel exhibited consistently high blood glucose levels. Post-administration of the first oral glucose dose, levels spiked to 600

mg/dL and then decreased to 435 mg/dL in 6 hours. The second glucose dose caused levels to rise back to 600 mg/dL, remaining elevated for the subsequent 6 hours, clearly indicating that diabetes was induced in test animals.

Treatment Group-1: Administration of the Actrapid Insulin (conventional formulation) injection in this group led to a significant decrease in blood glucose levels, reaching 71 mg/dL within 2 hours post the first oral glucose dose, and the normal blood glucose levels were maintained for 4 hours. However, levels increased after 4 hours, reaching 254 mg/dL at the 6-hour. When the second oral glucose dose was administered to the test animals in this group, the blood glucose levels rapidly escalated further and showed blood glucose levels similar to that of the placebo group at the 10-hour point and stayed consistent for 12 hours.

Treatment Group-2: This group, receiving the glucose-responsive hydrogel (study formulation), showed a distinctly different response from conventional insulin. After the initial oral glucose dose, blood glucose levels decreased gradually, normalizing after 6 hours. Following the second glucose dose, unlike Treatment Group-1, this group's blood glucose levels decreased after an initial rise, returning to normal levels similar to the control group at the 12-hour mark.

The comparative in-vivo efficacy study indicates that the study formulation can modulate insulin release effectively, providing extended glucose control compared to conventional insulin.

Even though the glucose-responsive formulation developed in this research showed a slower reduction in blood glucose levels compared to conventional insulin during the first 4 hours, the levels remained significantly below those observed in the placebo group. This initial lag can be attributed to the rapid in vivo gelation of the study formulation, which resulted in a slower initial decrease in blood glucose levels due to the lower availability of insulin compared to conventional insulin.

Moreover, following the second oral dose of glucose (after 6 hours), the blood glucose levels in treatment group 2 (receiving the glucose-responsive formulation) were comparably lower. They returned to normal by 12 hours post-administration. In contrast, treatment group 1 (receiving conventional insulin) exhibited elevated blood glucose levels similar to the placebo group.

These observations indicate that the glucose-responsive formulation developed in this research effectively modulates insulin release in response to fluctuating blood glucose levels, ensuring that insulin is released when needed, thus minimizing the risk of hypoglycemia. The reduced administration frequency and the hydrogel's glucose-responsive nature can enhance patient compliance and overall diabetes management. Furthermore, the hydrogel formulation provides a sustained insulin release, resulting in more stable and extended control of blood glucose levels, thereby reducing the frequency of insulin administration.

Glucose-responsive insulin delivery systems offer significant potential for improving diabetes management by automating insulin release in response to blood glucose levels, reducing the need for manual injections. Despite their

promise, several challenges need to be addressed. Insulin-releasing behaviors, especially in systems with high loading capacities, often lead to a continuous decline in insulin release after meals. This can result in hypoglycemia during initial doses and insufficient insulin supply as the system depletes. Additionally, balancing the frequency of insulin administration with the capacity of the delivery system is critical. Lower administration frequencies reduce pain and infection risk but require higher doses, which may harm cells at the administration site. Furthermore, the long-term biosafety of glucose-responsive systems remains uncertain due to potential toxicity from residual monomers and degradation products. Comprehensive studies are necessary to understand the metabolic pathways and ensure the safety of these systems [29]

Our glucose-responsive hydrogel formulation addresses these challenges by providing a sustained release of insulin, which ensures prolonged and stable control of blood glucose levels while reducing the frequency of administration. Unlike rapid-release systems that often require frequent administration, our hydrogel releases insulin gradually and modulates its release in response to fluctuating blood glucose levels. This minimizes the risk of hypoglycemia and ensures that insulin is available when needed, offering a more controlled glucose management solution. Additionally, our formulation uses biocompatible and biodegradable materials like chitosan and poloxamer, which reduces the risk of adverse reactions and enhances the long-term safety profile. The hydrogel's in situ formation and glucose-responsive behavior without complex chemical modifications make it practical for large-scale production and patient use.

We plan to address the initial lag time observed in blood glucose reduction further to optimize our glucose-responsive hydrogel formulation. This can be achieved by fine-tuning the insulin content and release kinetics of the hydrogel to ensure a more immediate response while maintaining the benefits of sustained release. Additionally, we aim to conduct comprehensive studies to assess the hydrogel's long-term bio-safety and metabolic pathways and its degradation products. These steps will help refine the formulation, enhancing its efficacy and safety for managing diabetes. With further optimization, particularly concerning the initial lag time and insulin content, our formulation promises to achieve more controlled and stable glucose levels in the diabetic population.

In conclusion, this research successfully developed a glucose-responsive insulin delivery system. With further optimization of insulin content, this formulation holds promise for achieving more controlled and stable glucose levels in the diabetic population.

4. Conclusion

This research successfully developed a novel glucose-responsive CS-PO hydrogel system for the subcutaneous delivery of insulin, demonstrating significant potential for diabetes management. The study meticulously optimized chitosan and Poloxamer 407 concentrations to achieve the desired sol-gel transition properties. This optimization was crucial in creating a hydrogel system that

responded effectively to pH and temperature changes, requiring a glucose-responsive delivery system.

The in vitro studies showed the hydrogel's ideal thermo-responsive and pH-sensitive characteristics. The hydrogel's solubilization rate varied with different pH levels and glucose concentrations, highlighting its potential for controlled insulin release. The injectability study confirmed the feasibility of administering this hydrogel through clinical-grade needles, a critical aspect of its practical application.

In vivo studies in diabetic rats revealed the hydrogel's glucose-responsive behavior. Compared to conventional insulin treatments, the hydrogel demonstrated a prolonged and more stable control of blood glucose levels. The hydrogel's rapid in vivo gelation and biodegradability, coupled with its ability to regulate insulin release in response to fluctuating glucose levels, marked a significant advancement over existing insulin delivery methods.

Furthermore, the hydrogel was fully biodegradable, with no adverse effects observed at the injection sites, underscoring its safety for clinical use. The study formulation showed promise in controlling multiple hyperglycemic episodes after a single dose, an advantage over conventional insulin therapies.

In conclusion, this research is significant in developing glucose-responsive insulin delivery systems. The CS-PO hydrogel system developed here offers a novel and effective approach to diabetes management, with the potential for further optimization to cater to the specific needs of the diabetic population. This system holds promise for enhancing the quality of life for individuals with diabetes by providing a more controlled and stable management of their blood glucose levels.

Conflict of interest

The authors declare to have no conflict of interest.

Acknowledgments

The authors thank Trustees Pune District Education Associations' Seth Govind Raghunath Sable College of Pharmacy, Saswad, Dist Pune, India, for the necessary facilities to complete this research.

References

[1] World Health Organization (WHO). Health Topics, Diabetes. https://www.who.int/health-topics/diabetes#tab=tab_1 (Accessed February 24, 2023).

[2] World Health Organization (WHO). Global Report on Diabetes. https://www.who.int/publications/i/item/9789241565257 (Accessed February 24, 2023).

[3] Yaturu S. Insulin therapies: Current and future trends at dawn. World J. Diabetes (2013) 4:1.

[4] Wang J, Wang Z, Yu J, Kahkoska AR, Buse JB, Gu Z. Glucose- Responsive Insulin and Delivery Systems: Innovation and Translation. Advanced Materials (2020) 32:1902004.

[5] Chou DH-C, Webber MJ, Tang BC, Lin AB, Thapa LS, Deng D, Truong JV, Cortinas AB, Langer R, Anderson DG. Glucose-responsive insulin activity by covalent modification with aliphatic phenylboronic acid conjugates. Proceedings of the National Academy of Sciences (2015) 112:2401–2406.

[6] Mansoor S, Kondiah PPD, Choonara YE. Advanced Hydrogels for the Controlled Delivery of Insulin. Pharmaceutics (2021) 13:2113.

[7] Mohanty AR, Ravikumar A, Peppas NA. Recent advances in glucose-responsive insulin delivery systems: novel hydrogels and future applications. Regen Biomater. https://doi.org/10.1093/rb/rbac056.

[8] Albin GW, Horbett TA, Miller SR, Ricker NL. Theoretical and experimental studies of glucose sensitive membranes. Journal of Controlled Release (1987) 6:267–291.

[9] Traitel T, Cohen Y, Kost J. Characterization of glucose-sensitive insulin release systems in simulated in vivo conditions. Biomaterials (2000) 21:1679–1687.

[10] Elshaarani T, Yu H, Wang L, Feng J, Li C, Zhou W, Khan A, Usman M, Amin BU, Khan R. Chitosan reinforced hydrogels with swelling-shrinking behaviors in response to glucose concentration. Int. J. Biol. Macromol. (2020) 161:109–121.

[11] Kumar MNVR, Muzzarelli RAA, Muzzarelli C, Sashiwa H, Domb AJ. Chitosan Chemistry and Pharmaceutical Perspectives. Chem. Rev. (2004) 104:6017–6084.

[12] Lee JI, Kim HS, Yoo HS. DNA nanogels composed of chitosan and Pluronic with thermo-sensitive and photo-crosslinking properties. Int. J. Pharm. (2009) 373:93–99.

[13] Yoo HS. Photo-cross-linkable and thermo-responsive hydrogels containing chitosan and Pluronic for sustained release of human growth hormone (hGH). J. Biomater. Sci. Polym. Ed. (2007) 18:1429–1441.

[14] Park KM, Lee SY, Joung YK, Na JS, Lee MC, Park KD. Thermosensitive chitosan–Pluronic hydrogel as an injectable cell delivery carrier for cartilage regeneration. Acta Biomater. (2009) 5:1956–1965.

[15] García-Couce J, Tomás M, Fuentes G, Que I, Almirall A, Cruz LJ. Chitosan/Pluronic F127 Thermosensitive Hydrogel as an Injectable Dexamethasone Delivery Carrier. Gels (2022) 8:44.

[16] Brambilla E, Locarno S, Gallo S, Orsini F, Pini C, Farronato M, Thomaz DV, Lenardi C, Piazzoni M, Tartaglia G. Poloxamer-Based Hydrogel as Drug Delivery System: How Polymeric Excipients Influence the Chemical-Physical Properties. Polymers (Basel) (2022) 14:3624.

[17] Schmolka IR. Artificial skin I. Preparation and properties of pluronic F-127 gels for treatment of burns. J. Biomed. Mater. Res. (1972) 6:571–582.

[18] Jeong B, Bae YH, Kim SW. Thermoreversible Gelation of PEG−PLGA−PEG Triblock Copolymer Aqueous Solutions. Macromolecules (1999) 32:7064–7069.

[19] Giovagnoli S, Tsai T, DeLuca PP. Formulation and Release Behavior of Doxycycline–Alginate Hydrogel Microparticles Embedded into Pluronic F127 Thermogels as a Potential New Vehicle for Doxycycline Intradermal Sustained Delivery. AAPS PharmSciTech (2010) 11:212–220.

[20] Kashyap N, Viswanad B, Sharma G, Bhardwaj V, Ramarao P, Ravi Kumar MNV. Design and evaluation of biodegradable, biosensitive in situ gelling system for pulsatile delivery of insulin. Biomaterials (2007) 28:2051–2060.

[21] OECD Test Guidelines for Chemicals (2001).

[22] Kim JS, Ju JB, Choi CW, Kim SC. Hypoglycemic and Antihyperlipidemic Effect of Four Korean Medicinal Plants in Alloxan Induced Diabetic Rats. Am. J. Biochem. Biotechnol. (2006) 2:154–160.

[23] Hatakeyama H, Hatakeyama T. Interaction between water and hydrophilic polymers. Thermochim. Acta (1998) 308:3–22.

[24] Gun'ko V, Savina I, Mikhalovsky S. Properties of Water Bound in Hydrogels. Gels (2017) 3:37.

[25] Zoldák G, Zubrik A, Musatov A, Stupák M, Sedlák E. Irreversible Thermal Denaturation of Glucose Oxidase from Aspergillus niger Is the Transition to the Denatured State with Residual Structure. J. Biol. Chem. (2004) 279:47601–47609.

[26] Cook MT, Haddow P, Kirton SB, McAuley WJ. Polymers Exhibiting Lower Critical Solution Temperatures as a Route to Thermoreversible Gelators for Healthcare. Adv. Funct. Mater. (2021) 31:2008123.

[27] Taylor M, Tomlins P, Sahota T. Thermoresponsive Gels. Gels (2017) 3:4.

[28] Kühl PW. Excess-substrate inhibition in enzymology and high-dose inhibition in pharmacology: a reinterpretation. Biochem. J. (1994) 298:171–180.

[26] Shen D, Yu H, Wang L, Khan A, Haq F, Chen X, Huang Q, Teng L. Recent progress in design and preparation of glucose-responsive insulin delivery systems. J. Control. Release (2020) 321:236–258.