Injectable microspheres for extended delivery of bioactive insulin and salicylic acid

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Abstract
Developing methods for insulin delivery continues to be of great translational research interest as insulin remains one of the most effective and commonly used treatments for diabetes. Bolus insulin injection at frequent intervals or insulin-loaded pumps used to treat diabetic patients have drawbacks including highly uneven kinetics, low patient compliance, enhanced chances of infections and disease transmission, and device fouling. This study evaluates the in vivo effects of insulin-loaded, salicylate-based, biocompatible, biodegradable polymeric microspheres that gradually release salicylic acid and insulin simultaneously. The study is predicated on the knowledge that such a continuous delivery system can release insulin over an extended period of time and overcome the aforementioned issues. Additionally, salicylic acid reduces insulin resistance in type-2 diabetic patients. In this work, we observed that insulin and salicylic acid were detected in serum over an extended period of time (at least 12 h and 4 days, respectively), and mice receiving insulin-loaded microspheres had a blood glucose reduction time frame ≥12 times that of bolus insulin administration.

Keywords
Insulin, biodegradable polymer, salicylic acid, sustained drug delivery, glucose levels

Introduction
Diabetes, a metabolic disorder caused by the resistance to and/or lack of insulin, is associated with numerous complications, including vulnerability to infections, peripheral neuropathy, high blood pressure, stroke, and impaired bone healing, and severely affects quality of life.¹,² The Centers for
Disease Control and Prevention reported an ~26 million diabetic population in the United States in 2011 and projected that one-third American adults will be diabetic by 2050 if the current trend continues.3

Insulin therapy is often an important part of diabetes treatment. A bolus of subcutaneously (s.c.) injected insulin is the most common form of delivery, yet it has several serious drawbacks. Notably, a sharp spike in serum insulin levels is accompanied by a steep decrease in blood glucose levels that can compromise patient safety. As insulin is rapidly metabolized, blood glucose levels can quickly rebound. To maintain glycemic control, patients have to inject insulin several times a day, which is not only extremely inconvenient but also increases the chances of infection and disease transmission. Insulin-loaded pH-sensitive hydrogels are also widely studied for oral insulin delivery. Typically, the hydrogel shrinks under acidic pH conditions and swells at neutral pH so that little insulin will be released in the stomach, whereas a burst release of insulin occurs in the intestine for absorption into the bloodstream.4 An oral delivery system is more convenient than subcutaneous injection yet suffers from the aforementioned bolus administration drawbacks. In addition, variability of oral insulin delivery is higher than injections so this system is not suitable for type-1 diabetic patients, where relatively precise insulin delivery is required.5

A sustained insulin delivery system is beneficial as it provides a relatively long time frame of insulin release, mitigating the need for frequent injections and the spike in blood insulin level. For many patients, insulin can be delivered via pumps. However, insulin pumps are plagued by higher costs, discomfort, restrictions on physical activity, device fouling, and other issues.6–8

Insulin-loaded, biodegradable polymeric microspheres are a promising alternative for sustained insulin delivery. As the polymer gradually degrades after injection, the encapsulated insulin will be released in a sustained manner. Unreleased insulin is shielded by the polymer matrix and thus protected from enzymatic degradation. However, most biodegradable polymers used to form insulin-loaded microspheres, such as poly(lactic-co-glycolic acid) (PLGA), poly ε-caprolactone (PCL), poly(lactic acid) (PLA), and chitosan, act only as a drug carrier with no bioactive degradation products.

In our previous work, insulin-encapsulating microspheres were formulated using a salicylic acid–based poly(anhydride ester) (SAPAE). SAPAE hydrolyzes into an anti-inflammatory drug salicylic acid (SA), so the microspheres concurrently delivered two drugs—SA and insulin—as the SAPAE degraded.9 The combinatory delivery of insulin and SA is of particular clinical significance as a number of studies have demonstrated that salicylates, a family of anti-inflammatory drugs, can improve glucose metabolism in diabetic patients by ameliorating insulin resistance.10–13 A clinical study showed that high-dose aspirin reduced ~25% of fasting plasma glucose and ~30% insulin clearance in type-2 diabetic patients.10 This phenomenon results because salicylate can ameliorate the chronic inflammation associated with diabetes, which has been found to aggravate insulin resistance in diabetic patients.14 At the molecular level, IκB kinase-β (IKK β) and nuclear factor (NF)-κB, which lead to insulin resistance, are upregulated in diabetes and salicylates can inhibit the activity of both molecules, therefore, ameliorating insulin resistance.14–17

We previously tested the in vitro activity of the insulin and SA released from microspheres, demonstrating that SA reduced tumor necrosis factor-α (TNF-α) secretion from lipopolysaccharide (LPS)-activated macrophages, whereas the insulin increased phosphorylated Akt level in rat myoblasts.9 This study deepens the understanding of dual SA/insulin delivery and further investigates the in vivo pharmacokinetics of this dual delivery system and its effect on blood glucose levels. Both SA and insulin are detected in mice sera over an extended period of time (at least 4 days and 12 h, respectively). Importantly, the released insulin is bioactive and has a therapeutic window that is ≥12 times longer than bolus insulin treatment.
Materials and methods

Materials

All chemicals and reagents, including insulin and SA, were purchased from Sigma–Aldrich (Milwaukee, WI, USA) and used as received.

Microspheres formation and characterization

Formation and characterization of microspheres were performed based on the previously published method. Briefly, 10 mg of insulin was suspended in 100 µL of phosphate buffered saline (PBS) and mixed with 200 mg of SAPAE dissolved in 2 mL of dichloromethane (DCM). The mixture was homogenized for 1 min at ~15,000 r/min using an ULTRA-TURRAX T8 Homogenizer (IKA, Wilmington, NC, USA). The resulting emulsion was added dropwise to 100 mL of 1% (w/v) poly(vinyl alcohol) (PVA, 80% hydrolyzed, 30–70 kDa) and homogenized for 4 min at ~10,000 r/min and stirred for 3 h at 500 r/min at room temperature to evaporate DCM. The microspheres were precipitated by centrifugation at 3000 r/min for 5 min. After centrifugation, the microspheres were washed 4–5 times with deionized water (pH 7.0) to remove excess PVA, lyophilized in a FreeZone® 4.5 Freeze Dry System (Kansas City, MO, USA) overnight, and stored at −20°C before use. Nonloaded microspheres, which act as negative control, were prepared following the same protocol without the addition of the insulin. Microsphere formation and diameter size were confirmed by scanning electron microscopy, as previously published.

In vivo study

All animal studies were approved by the Angion Biomedica Institutional Animal Use and Care Committee. Nonfasting, adult female C57BL/6 mice (Charles River Labs, Wilmington, MA, USA) (~20 g, n = 5) were administered insulin (50 mg/kg, s.c.) and blood (tail vein) samples monitored at the time points described below. Each mouse, therefore, served as its own baseline control. For the microsphere studies, nonfasting adult female C57BL/6 mice (~20 g, n = 5) were administered SAPAE (1250 mg/kg, s.c.) or SAPAE + insulin (1250/50 mg/kg, s.c.) and blood samples monitored at the time points described below. For measurements of serum SA or insulin, animals (n = 3) were anesthetized (ketamine/xylazine 50/5 mg/kg, intraperitoneal) at the time points described below and serum samples obtained. Another group of C57BL/6 mice was administered SA only (450 mg/kg, s.c.) as positive control. At the end of the study, mice were sacrificed via asphyxiation with CO₂ and/or exsanguination.

In vivo insulin concentration determination

Serum insulin levels were quantified using an ALPCO Diagnostics enzyme-linked immunosorbent assay (ELISA) kit (Catalog #80-INSMS-E01, E10; Salem, NH, USA) according to the manufacturer’s instructions.

In vivo SA concentration determination

Serum SA levels were quantified using a Salicylates ELISA assay kit (Neogen Corporation, Lexington, KY, USA) according to the manufacturer’s instructions.
Blood glucose determination

Blood glucose was monitored from mouse tail vein samples using a glucometer (OneTouch Ultra, Garden City, NY, USA).

Data analyses

Data are expressed as mean ± standard error. Average blood glucose at any time point was compared to the average blood glucose at baseline using a $t$-test. A $p$ value <0.05 was considered significant.

Results and discussion

The goal of this study was to study the in vivo pharmacokinetics and bio-activity of the insulin-loaded SA-based polymeric microspheres. We observed that the loaded microspheres have well-defined spherical shapes and smooth surface. Both SA and insulin were released from the microspheres in a sustained manner, and the dual delivery system was effective in reducing blood glucose level in diabetic rats for an extended period of time compared to a bolus insulin injection.

Microsphere surface morphology

Successful microsphere formation was achieved as shown by the smooth spherical geometry of both insulin-loaded and nonloaded SAPAE microspheres (Figure 1). No broken spheres were observed, and microsphere geometry, surface smoothness, and sizes agree with previous work.9 In general, all microspheres displayed a smooth surface and sizes of ~13 µm (12.3 ± 6.4 µm for insulin-loaded and 13.9 ± 7.6 µm for nonloaded microspheres).

In vivo SA release profile

Nonloaded SAPAE microspheres (with no insulin encapsulated) gradually released SA over 2 days in mice (Figure 2), and no significant adverse effects were observed, whereas the equivalent bolus SA injection (450 mg/kg, s.c., positive control) resulted in a sharp and steep increase in serum SA concentration (up to 1623 µg/mL) at 8 h. The SA bolus contains the same SA concentration as
SAPAE, yet beyond this time point, none of the SA bolus mice survived the administration likely due to systemic SA toxicity. These data suggest that controlled and extended SA release from SAPAE that occurs via polymer degradation is safe and tolerable.

The in vivo SA release profile from SAPAE generally agrees with the established in vitro release profile, that is, a lag period of SA release (little-to-no SA released) is followed by a sustained SA release. Notably, the release window of SA in vivo is shorter than in vitro, which is likely due to both enhanced metabolism and clearance of SA in vivo. 

**Sustained glucose reduction**

Blood glucose concentrations in nonfasting mice receiving bolus insulin injection returned to basal level after only 1.5 h, whereas mice receiving insulin-encapsulated SAPAE microspheres have significantly reduced blood glucose level even after 12 h (Figure 3, left). Blood glucose was not tested between 12 and 24 h, and it is plausible that blood glucose remained depressed in these non-fasting mice beyond 12 h. By 24 h, blood glucose had returned to normal levels indicating that the system releases bioactive insulin whose effects are reversible. None of the mice exhibited any signs of gross toxicity, observations consistent with the SA pharmacokinetics study reported above.

Mice injected with unloaded microspheres did not display significant blood glucose reduction, supporting the notion that the blood glucose reduction is attributed to insulin and not SAPAE or SA. These data suggest that bioactive insulin is released from SAPAE microspheres in a sustained manner and, impressively, the system has $\geq 12$ times longer effective therapeutic window compared to a bolus insulin delivery (Figure 3, right).

**Correlation between glucose level and serum insulin concentration**

To confirm the inverse relation between blood glucose levels and serum insulin concentration, serum insulin was quantified 5 h after injection (when a significant blood glucose reduction was observed) as well as 24 h post-injection (when blood glucose returned to the basal level). At 5 h, serum insulin level was high and blood glucose was low, whereas the opposite trend was observed at the 24-h time point (Figure 4). These data indicate that blood glucose levels are inversely correlated with serum insulin concentration, confirming that the reduction of blood glucose is primarily due to sustained insulin release from the microspheres.
Conclusion

This work reports the first in vivo pharmacokinetic data of insulin-loaded SAPAE microspheres and correlates with the established in vitro data. Sustained release of both insulin and SA from the microspheres was observed in vivo. Insulin activity was well maintained after microsphere formulation/processing, and its functional time frame was greatly extended by SAPAE encapsulation. These data indicate the potential of this system to treat diabetes and the associated inflammation. An attractive feature for use of SA as an anti-inflammatory is that unlike aspirin (aka acetyl salicylic acid), SA does not have blood-thinning capabilities, that is, it is not anti-thrombogenic. Future studies will be performed to extend the window of insulin and SA release to a more patient-friendly span, that is, from hour/days to weeks/months. By combining two drugs that treat diabetes and have demonstrated synergistic effects, the insulin-loaded SAPAE system can change the paradigm for biologics in clinical medicine perhaps paving the way for use of other therapeutic peptides in an extended release format.

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Declaration of conflicting interests

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References