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Biodegradable Salicylate-Based Poly(anhydride-ester) Microspheres For Controlled Insulin Delivery

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Abstract

Salicylate-based poly(anhydride-esters) (PAEs) chemically incorporate salicylic acid (SA) into the polymer backbone, which is then delivered in a controlled manner upon polymer hydrolysis. In this work, a salicylate-based PAE is a carrier to encapsulate and deliver insulin. Polymer microspheres were formulated using a water/oil/water double-emulsion solvent evaporation technique. The microspheres obtained had a smooth surface, high protein encapsulation efficiency, and relatively low emulsifier content. Insulin was released *in vitro* for 15 days, with no signs of aggregation or unfolding of the secondary structure. The released insulin also retained bioactivity *in vitro*. Concurrently, SA was released from the microspheres with polymer degradation and anti-inflammatory activity was observed. Based upon these results, the formulated microspheres enable simultaneous delivery of insulin and SA, both retaining bioactivity following processing.

Keywords

poly(anhydride-esters); microspheres; controlled release; insulin; salicylic acid

1. Introduction

Proteins are commonly used therapeutics and their delivery can have great impact on their therapeutic efficacy and efficiency.¹ Bolus administration is often used due to its simplicity in practice, but the resulting protein concentration in the body may increase rapidly and lead to undesirably high drug concentration that is toxic to the patient.² Often, the protein concentration will quickly decrease to a non-effective level, thus frequent dosing is required to maintain an effective protein concentration over a long time period.²

Controlled protein delivery overcomes the aforementioned problems by maintaining a relatively constant protein release rate, such that protein concentration stays in the therapeutic range for a longer time.³ Polymeric biodegradable microspheres are broadly used for controlled protein delivery. They are advantageous as they can be injected to avoid

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invasive implantation.⁴ Moreover, polymer accumulation *in vivo* is negligible because it is biodegradable and the degradation products can be cleared from the body. In addition, as protein is encapsulated within the microspheres before release, it is protected from enzymatic denaturation, which is crucial to retain the protein bioactivity.^{5,6}

Among different biodegradable polymers, poly(lactic-*co*-glycolic) acid (PLGA), is extensively used for controlled protein delivery.^{7–11} Despite its popularity and approval by the Food and Drug Administration in multiple medical devices, concerns remain about the denaturation of encapsulated proteins due to the decrease in pH with polymer degradation.⁵ Furthermore, it has been reported that PGLA implantation can enhance inflammation at the site.¹²

As an alternative to PLGA and other biodegradable polymers, we propose a salicylate-based poly(anhydride-ester) (PAE) to encapsulate and deliver proteins. This polymer chemically incorporates salicylic acid (SA) into the polymer backbone (Figure 1).^{13,14} It is biocompatible, hydrolytically degradable, and releases SA in a near zero-order manner.¹⁴ Furthermore, this polymer mitigates inflammation when implanted *in vivo*.¹⁵ Salicylate-based PAEs have been used as carriers to deliver small bioactive molecules, such as antimicrobials¹⁶ and other small compounds, and have been formulated into microspheres.^{17,18} We propose that salicylate-based PAE microspheres can encapsulate and deliver larger molecules, such as proteins. In this work, we encapsulate insulin as model protein and achieve controlled insulin release *in vitro*.

Insulin-loaded polymer microspheres were formulated using a water/oil/water (w/o/w) double-emulsion solvent evaporation technique. The size and morphology of the microspheres were studied using scanning electron microscopy (SEM). Protein loading and encapsulation efficiency were determined; the amount of residual emulsifier [poly(vinyl alcohol) (PVA)] was quantified; and the weight-average molecular weight (M_w) and glass transition temperature (T_g) of the polymer once formulated into microspheres were compared to the unprocessed polymer. Insulin and SA *in vitro* release profiles were studied. Structural integrity of released insulin was studied using native polyacrylamide gel electrophoresis (native-PAGE) and circular dichroism (CD) spectroscopy. *In vitro* bioactivity of the released insulin was measured by monitoring insulin-induced protein kinase B (Akt) phosphorylation level. In addition, SA activity was measured by the reduction of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF- α) secretion by human blood-derived macrophages *in vitro*.

2. Materials and Methods

2.1 Materials

Phosphate buffer saline (PBS, pH 7.4) was purchased from Mediatech (Herndon, VA). Micro bicinchoninic acid (BCA) protein assay kit and silver stain kit were purchased from Pierce (Rockford, IL). ELISA kit against human TNF- α was purchased from BioLegend (San Diego, CA). L6 rat myoblast cells were purchased from ATCC (Manassas, VA). Fast activated cell-based ELISA (FACETM) Akt kit was purchased from Active Motif (Carlsbad, CA). RPMI-1640 media, trypsin-EDTA, penicillin/streptomycin, L-glutamine and fetal

bovine serum were purchased from Gibco (Grand Island, NY). Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon-gamma (IFN-gamma) were purchased from R&D Systems (Minneapolis, MN). Human blood from healthy donors was purchased from the Blood Center of New Jersey. Ficoll was purchased from GE healthcare (Piscataway, NJ). All other chemicals and reagents were purchased from Sigma-Aldrich (Milwaukee, WI) and used as received.

2.2 Microspheres preparation

Salicylate-based PAE was synthesized following previously published methods.^{13,14} Polymer properties were as follows: $M_w = 20,000$ Da and $T_g = 49^\circ\text{C}$. Insulin-loaded microspheres were prepared from salicylate-based PAE by a w/o/w double emulsion solvent evaporation technique: 10 mg of insulin was suspended in 100 μL of PBS and mixed with 200 mg of polymer dissolved in 2 mL of dichloromethane (DCM). The mixture was homogenized for 1 min at $\sim 15,000$ rpm using an ULTRA-TURRAX T8 homogenizer (IKA, Wilmington, NC). The resulting emulsion was added dropwise to 100 mL of 1% (w/v) PVA (80 % hydrolyzed, 30–70 kDa), homogenized for 4 min at $\sim 10,000$ rpm, and stirred for 3 h at 500 rpm at room temperature to evaporate DCM. The microspheres were precipitated by centrifugation at 3000 rpm for 10 min. After centrifugation, the microspheres were washed several times with deionized water (pH 7.0) to remove excess PVA indicated by few bubbles present after shaking, lyophilized in a Freezone® 4.5 Freeze Dry System (Kansas City, MO) overnight, then stored at -20°C before use. Non-loaded microspheres, a negative control, were prepared following the same protocol without the insulin addition.

2.3 Microspheres size and surface morphology

Microspheres (~ 5 mg) were distributed on aluminum studs, sputter-coated with gold-palladium using a SCD 004 Sputter Coater (Bal-Tec, Canonsburg, PA) and imaged with an AMRAY1830 I scanning electron microscope. To determine the size of the microspheres, several representative SEM images were analyzed using NIH ImageJ Software. Values obtained from single microsphere measurements ($n > 100$) were averaged to obtain the mean particle size distribution for both formulations, i.e., non-loaded and insulin-loaded microspheres.

2.4 Thermal analysis

Thermal analysis was performed using differential scanning calorimetry (DSC) to obtain T_g values. DSC was performed using a Thermal Advantage (TA) DSC Q200 (TA Instruments, New Castle, DE) running on an IBM ThinkCentre computer equipped with TA Instrument Explorer software for data collection and TA Universal Analysis 2000 version 4.5A for data processing. Samples were prepared by weighing about 4 to 8 mg of sample into an aluminum pan, and hermetically sealed. The samples were heated under nitrogen from -20°C to 160°C at a rate of $10^\circ\text{C}/\text{min}$. A minimum of two heating cycles was used with T_g defined as the curve midpoint.

2.5 Molecular weight

Gel permeation chromatography (GPC) was used to determine the weight-averaged M_w of the polymer before and after formulation. A Perkin-Elmer LC system (Perkin Elmer, Waltham, MA) consisting of a Series 200 refractive index detector, a Series 200 LC pump, and an ISS 200 advanced sample processor was used. A Dell OptiPlex GX110 computer running Perkin-Elmer TurboChrom 4 software was utilized for data collection. The connection between the LC system and the computer was a Perkin-Elmer Nelson 900 Series Interface and 600 Series Link. Samples were dissolved in DCM (10 mg/mL) and filtered through 0.45 μ m polytetrafluoroethylene (PTFE) syringe filters (Whatman, Clifton, NJ) prior to elution through a Jordi divinylbenzene mixed-bed GPC column (7.8×300 mm) (Alltech Associates, Deerfield, IL) at a rate of 1 mL/min for a total run time of 30 min. In the case of the insulin-loaded microspheres, the polymer was separated from the insulin following the extraction procedure discussed in the protein content determination section. M_w was calculated relative to narrow M_w polystyrene standards (Polysciences, Dorval, Canada).

2.6 Residual PVA determination

Residual PVA in the microspheres was determined by a colorimetric assay based on the formation of a complex between the hydroxyl groups in PVA and iodine in the presence of boric acid.¹⁹ Briefly, 2 mL of 0.5 N NaOH was added to 2 mg of the freeze-dried microspheres (non-loaded and insulin-loaded) and incubated for 15 min at 60 °C. To each sample, 900 μ L of 1 N HCl were added and the total volume adjusted to 5 mL with deionized water. To this sample, 3 mL of 0.65 M boric acid was added, followed by 0.5 mL of 0.05M/0.15M I_2 /KI solution and 1.5 mL of deionized water, and then held at room temperature for 15 min. Absorbance was measured at 690 nm using a Perkin Elmer Lambda XLS UV/vis spectrophotometer (Perkin Elmer, Waltham, MA). A UV/vis calibration curve was prepared using PVA solutions with known concentrations. Experiments were performed in triplicate.

2.7 Protein content determination

Insulin loading [(mass of insulin in microspheres/mass of microspheres) $\times$ 100 %] and encapsulation efficiency [(measured insulin loading/theoretical insulin loading) $\times$ 100 %] were determined by quantifying encapsulated insulin in microspheres. Then, 10 mg of insulin-loaded microspheres was dissolved in 500 μ L of DCM, 500 μ L of 0.1 N HCl was added and vortexed for 1 min to extract insulin. The mixture was then allowed to settle for 10 min before the aqueous phase was collected for further quantification. Protein quantification was performed using the Micro BCA assay according to manufacturer's protocol. Same treatments were performed on non-loaded microspheres as a negative control. Experiments were performed in triplicate.

2.8 *In vitro* release studies of SA and insulin

In vitro release studies were performed by placing 10 mg of non-loaded or insulin-loaded microspheres in scintillation vials (Fisher, Fair Lawn, NJ) with 10 mL of PBS. Samples were incubated at 37 °C with agitation (60 rpm) using a controlled environment incubator-

shaker (New Brunswick Scientific Co., Edison, NJ) for 30 days. At predetermined time points, 8.5 mL aliquots were withdrawn and replaced with 8.5 mL of fresh PBS for insulin-loaded microspheres; 5 mL aliquots were withdrawn and replaced with 5 mL of fresh PBS for non-loaded microspheres. To quantify SA released from microspheres, aliquots were analyzed using a Perkin Elmer Lambda XLS UV/vis spectrophotometer at 303 nm, which is the maximum absorbance of SA that does not overlap with the UV absorbance of other degradation products. The cumulative % SA released at each time point was normalized against the total SA released during the 30-day period. Insulin released at each time point was determined with the Micro BCA assay and normalized against the vehicle control (non-loaded microspheres) to eliminate possible absorbance from the SA. The cumulative % insulin released at each time point was normalized against the total insulin loading. Experiments were performed in triplicate.

2.9 Structural integrity of insulin

Native polyacrylamide gel electrophoresis (native-PAGE) was performed using a Mini-PROTEAN® Tetra electrophoresis system (Bio-Rad, Hercules, CA). Samples were collected from *in vitro* release media at day 7, filtered using a 0.45 µm syringe filter (Corning, Corning, NY) and mixed 2:1 with sample buffer (20% glycerol, 0.001% bromophenol blue and 0.5 M Tris-HCl). Samples were separated on a 15% polyacrylamide gel and the electrophoresis was performed at constant voltage (120 V) for 3.5 h. Insulin released from the microspheres was compared against the unprocessed insulin (freeze-dried powder diluted in PBS and mixed 2:1 with sample buffer). Gels were stained using a silver stain kit according to manufacturer's protocol and immediately photographed after development. Experiments were performed in triplicate.

CD spectroscopy was performed on an Aviv Model 400 CD Spectropolarimeter (Aviv Biomedical, Inc., Lakewood, NJ) that was calibrated with 10-camphorsulfonic acid at 290.5 nm. Samples were collected from *in vitro* release media at day 7 and filtered with a 0.45 µm syringe filter. Standard solutions of unprocessed insulin and the respective release samples were prepared at protein concentrations of approximately 20 µM in a buffer system comprised of PBS and 150 mM sodium chloride adjusted to pH 7.0. To remove traces of SA, solutions were buffer-exchanged using ultrafiltration (Amicon® Ultra 4mL Filters for Protein Purification and Concentration). The samples were transferred to 0.1 cm quartz cuvettes and equilibrated at 25 °C for 10 min prior to optical analysis. The protein conformation was evaluated by recording the ellipticity values (θ , units of deg cm² dmol⁻¹) over the wavelength range of 200 to 250 nm at 1.0 nm increments following signal averaging for 15 seconds using conventional procedures. Experiments were performed in triplicate.

2.10 L6 rat myoblast cell culture

L6 rat myoblasts were used to evaluate the *in vitro* insulin activity. Rat L6 myoblasts were cultured in T75 flasks containing 15 mL complete DMEM media supplemented with 20% FBS and 1% penicillin/streptomycin. Cells were incubated at 37°C with 5% CO₂ under atmospheric pressure. When cells reached 80% confluence, the media was aspirated and cells detached with 4 mL trypsin-EDTA solution, then incubated for 5 min. Trypsin was

neutralized with 5 mL of complete media, and cells were centrifuged at 200×g for 5 min. After centrifugation, supernatant was removed and the cell pellet was resuspended in 1mL of complete media. Cells were counted using hemocytometer under an inverted microscope (Olympus, Melville, NY).

2.11 *In vitro* insulin bioactivity: FACE Akt test

L6 myoblast cell suspensions were diluted to 112,500 cells/mL in complete media and 200 µL per well was added to a 96 well plate supplied in the FACE Akt kit. Cells were incubated at 37°C with 5% CO₂ under atmosphere for 24 h, washed twice with 200 µL PBS, supplied with 200 µL starvation media (99% DMEM and 1% penicillin/streptomycin), and incubated for another 6 h. Cells were then stimulated with: (1) insulin-loaded microsphere degradation media from day 1 (denoted as DM1), 4 (DM4) and 12 (DM12), then diluted with starvation media to a final concentration of 150 nM insulin; (2) unprocessed insulin dissolved in PBS and then diluted with starvation media to a final concentration of 150 nM (denoted as UI); and (3) PBS diluted with starvation media (PBS). All samples have the same concentration of PBS (23.5%) to eliminate possible effect of PBS on the results. After 20 min stimulation, wells were processed according to the FACE Akt kit protocol, including cell density quantification and phosphorylated Akt normalization. Six replicates were performed for each group.

2.12 Human blood-derived monocyte isolation and macrophage differentiation

Human blood-derived monocytes were used to study microsphere cytocompatibility *in vitro* and determine the anti-inflammatory activity of SA released from the polymer microspheres. Cell isolation and purification methods used were previously described by Kim *et. al* (2009).²⁰ Briefly, peripheral blood mononuclear cells were collected from blood from healthy donors (Blood Center of New Jersey) by density gradient separation using ficoll at a density of 1.077. Red blood cells were lysed by incubation in ACK lysis buffer for 5 min, washed with PBS, and counted. Monocytes were cultured on 175 cm² flasks (BD, Franklin Lakes, NJ) at a concentration of 8×10^6 cells/mL in RPMI-1640 media (GIBCO BRL, Rockville, MD). RPMI media was supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 4 mM L-glutamine. Monocytes were allowed to adhere for 2 h, then washed 3 times with PBS to remove non-adherent cells. Monocytes were cultured for 7 days at 37 °C and 5% CO₂ in RPMI supplemented with 5 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) to generate macrophages. At day 6, interferon-gamma (IFN-gamma) (25 ng/mL) was added to the cell culture flask. After 7 days of culture, macrophages were washed once with PBS, then detached with trypsin-EDTA for 30 min at room temperature. Cells were re-suspended in RPMI, counted, re-plated at 5×10^4 cells/well of a 24 well plate (NUNC-Thermo Scientific, Rochester, NY) and allowed to attach overnight. The following day, cells were used for experiments.

2.13 *In vitro* TNF-α inhibition

Human blood-derived monocytes were utilized to monitor the inhibition of TNF-α in the presence of SA released from the polymer microspheres. TNF-α secretion was achieved by activation of the macrophages with lipopolysaccharides (LPS) (1 µg/mL). During the cell

culture process, the macrophages were primed with the macrophage activation factor IFN- γ . This activation factor enhances the production of inflammatory cytokines from macrophages once exposed to LPS. Macrophages were seeded at a density of 5×10^4 cells/well in a 24 well plate containing 500 μ L of culture medium. After LPS activation, a Transwell® Permeable Support (8.0 μ m polycarbonate membrane) (Costar, Corning, NY) was placed on top of the monocytes and 200 μ L of non-loaded microspheres suspended in solution (sterile PBS) added to the inserts at two different concentrations: 100 μ g/mL and 500 μ g/mL. Cells were incubated at 37 °C and 5% CO₂ for 48 h. After 48 h, conditioned media was collected and TNF- α secretion was determined with an ELISA kit against human TNF- α according to manufacturer's protocol. A student's t-test was performed to determine significant differences between the positive control (media with LPS only) and the experimental conditions. Experiments were performed in quadruplicate.

3. Results and Discussion

In this work, we demonstrate that salicylate-based PAE microsphere encapsulated and delivered insulin, as well as simultaneously released SA that was chemically incorporated into the polymer backbone. Non-loaded microspheres were used as the negative control. The resulting microspheres were characterized to assess their size, morphology, residual PVA content, T_g , M_w , protein content, *in vitro* release profiles, cytocompatibility, released protein structure, and anti-inflammatory activity.

The w/o/w double-emulsion solvent evaporation technique yielded microspheres with smooth surfaces (Figure 2). Both sets of microspheres, non-loaded and insulin-loaded, had average diameters of ~ 25 μ m (Table 1). When preparing the microspheres, a goal of 5 wt% of insulin was intended in the formulation and ~ 4.1 wt% was achieved; the encapsulation efficiency was calculated to be 82.6 % (Table 1). The amount of residual PVA was ~ 3.9 μ g/mg microsphere for non-loaded microspheres and ~ 3.4 μ g/mg microsphere for insulin-loaded (Table 1), this minimal PVA level should have minimal impact on the microsphere performance.²¹

The T_g values of the microspheres were compared to the values obtained for the unprocessed polymer (49 °C). The T_g for the non-loaded microspheres was 37 °C and 55 °C for insulin-loaded microspheres (Table 1); both the non-loaded and insulin-loaded microspheres had T_g values at or above 37 °C, ensuring their structural integrity *in vivo*.

M_w was investigated before and after microsphere formulation to determine how the formulation affects the polymer. Before formulation, the polymer M_w was 20,000 Da and decreased 14% and 23% for the non-loaded and insulin-loaded microspheres, respectively (Table 1). This result is expected as the hydrolytically degradable polymer was exposed to water during formulation and would be degraded to some extent. It has been previously shown that the decrease in M_w of the salicylate-based PAE during microsphere formulation is reduced by washing the microspheres with acidic water (only 7 % decrease in M_w).²² However, exposure to acidic water could potentially affect the insulin integrity, thus the microspheres were washed with distilled water at neutral pH.

In vitro release studies were performed for 30 days to determine the insulin and SA release profiles. The study was performed under physiological conditions (pH 7.4, 37 °C, and agitation) to gain an understanding about how this system would perform *in vivo*. A burst of insulin was observed on day 1 with 45 % of the protein released, likely due to insulin present on the microsphere surface. After the initial 1-day burst, the remaining insulin was gradually released for 15 days. SA release profiles of insulin-loaded and non-loaded microspheres were similar, and complete SA release was achieved in 30 days (Figure 3).

When delivery systems are formulated, the protein stability is a major concern because the protein is often exposed to organic solvents, emulsifiers, and the polymer as a carrier.²³ Proteins can denature, unfold, and/or aggregate resulting in the loss of biological activity. Thus, it is important to determine if the structural integrity of the protein changes in the formulation and delivery processes. Native-PAGE was performed to monitor insulin released from microspheres at day 7 (chosen for proof-of-concept), specifically to detect protein aggregation or unfolding upon release from the polymer microspheres. Unprocessed insulin showed a single band in the gel; similar bands were observed for insulin released from the salicylate-based PAE microspheres (Figure 5A). No high M_w or low M_w bands were detected, suggesting that the released insulin at day 7 maintains its primary structural integrity. To evaluate the secondary structure of the insulin released from the salicylate-based PAE microspheres, CD spectroscopy was performed. The CD spectrum of the unprocessed insulin standard exhibits two minima at 208 and 223 nm, corresponding to the α -helix in insulin. This pattern is observed for the insulin released from the polymer microspheres (Figure 5B). The two spectra are virtually identical, suggesting that the secondary structure of insulin in both samples (unprocessed and released insulin) is comparable within experimental error. The observed difference is presumably due to the uncertainty associated with protein concentration determination in the presence of residual amounts of UV-absorbing SA.

To further confirm that insulin was not denatured during microsphere formulation and the release process, FACE Akt test was used to validate the *in vitro* bioactivity of insulin. Insulin will induce Akt phosphorylation such that the intracellular level of phosphorylated Akt will be alleviated upon insulin stimulation, which can be determined through the FACE Akt test. The phosphorylated Akt level was normalized with unprocessed insulin (UI) and expressed as a percentage. All degradation media groups have significantly higher insulin activity compared to the negative control (PBS), indicating that the released insulin retained most of its bioactivity. However, a slight decrease in insulin activity was observed over time by comparing samples of DM1 with DM4 and DM12, suggesting that insulin activity was slightly affected during the release process; this result is consistent with studies reported in the literature.²⁴ Further, SA in the degradation media could have synergistic effect with insulin and enhance the activity of insulin.²⁵

The salicylate-based PAE releases SA in a controlled manner; however, the *in vitro* anti-inflammatory activity of SA has not been directly tested to date. Therefore, an inflammatory assay was performed to monitor the inflammation cytokine TNF- α secretion in the presence of SA released from the polymer microspheres. TNF- α is a cytokine from the TNF superfamily involved in systemic inflammation and secreted by a variety of cells in the

body, including macrophages. Therefore, reduction of TNF- α secretion correlates with anti-inflammatory activity. LPS-activated macrophages were exposed to two different concentrations of non-loaded microspheres (100 $\mu\text{g/mL}$ and 500 $\mu\text{g/mL}$) for 48 h. Non-loaded microspheres were used to detect anti-inflammatory activity produced by SA alone, and not by insulin or SA-insulin combination. Microspheres at a concentration of 100 $\mu\text{g/mL}$ resulted in minimal decrease in TNF- α secretion at 48 h when compared to the positive control (Figure 6). In contrast, microspheres at a concentration of 500 $\mu\text{g/mL}$ showed statistically significant inhibition (approximately 20% decrease) of TNF- α secretion (Figure 6). These results demonstrate that SA released from the microspheres retained their anti-inflammatory activity.

4. Conclusion

A salicylate-based PAE was formulated into microspheres encapsulating insulin. This system simultaneously released insulin and SA in a controlled and sustained manner. Both insulin and SA retained their *in vitro* activity after the formulation process and release. This work has great potential in controlled insulin delivery, especially towards diabetic patients due to the synergistic effects of SA and insulin.²⁵ In addition, this work lays the foundation for encapsulating other therapeutic proteins using salicylate-based PAE. Future works include optimizing the bioactive release profile to match different applications and *in vivo* tests.

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Figure 1. Hydrolytic degradation of salicylate-based PAE into salicylic acid (SA) and the adipic acid linker.

Figure 2. SEM images of non-loaded (A) and insulin-loaded (B) salicylate-based PAE microspheres. Both sets of microspheres display similar sizes and morphology (spherical shape and smooth surface).

Figure 3.
In vitro insulin release profile from insulin-loaded microspheres ($\pm$ standard deviation).

Figure 4.
In vitro SA release profiles from insulin-loaded microspheres and non-loaded microspheres ($\pm$ standard deviation).

Figure 5.

(A) Native-PAGE analysis of insulin released from salicylate-based PAE microspheres at day 7 (lane 1 standard is molecular marker, lane 2 is blank, lane 3 is unprocessed-insulin, and lanes 4–6 are insulin released from salicylate-based PAE microspheres *in vitro*). (B) Far-UV CD spectra of insulin standard and insulin released from salicylate-based PAE microspheres *in vitro*.

Figure 6.

Insulin activity determined by quantifying phosphorylated Akt level upon different stimulations. Numbers were normalized with unprocessed insulin. Samples include: unprocessed insulin (UI) diluted in starvation media; *in vitro* degradation media from insulin-loaded microspheres diluted in starvation media: DM1 (degradation media from day 1), DM4 (degradation media from day 4), and DM12 (degradation media from day 12); PBS diluted in starvation media as negative control. All samples contain 150nM insulin (except the PBS sample).

Figure 7. TNF- α secretion levels from blood derived macrophages cultured with non-loaded salicylate-based PAE microspheres. * $P < 0.05$ relative to the positive control (LPS only).

Table 1

Physicochemical properties of the non-loaded and insulin-loaded salicylate-based PAE microspheres.

	Non-loaded Microspheres	Insulin-loaded Microspheres
Mean particle size (μm)	25 ± 8	25 ± 10
T_g ($^{\circ}\text{C}$)	37	56
M_w after formulation (Da)	17,200	15,300
Residual PVA ($\mu\text{g}/\text{mg}$ microspheres) *	3.9 ± 0.5	3.4 ± 0.9
Protein loading (% w/w)	-	4.1 ± 0.6
Encapsulation efficiency (%) *	-	82.6 ± 12.1

** Mean $\pm$ standard deviation for $n > 100$;

* Mean $\pm$ standard deviation for $n = 3$.

Initial polymer M_w was 20,000 Da. Particle size was measured by analysis of SEM images using NIH Image J Software.